ผลของเชื้อโนซีมาที่สกัดจากผึ้งไทยต่อความเครียดทางด้านพลังงานในผึ้งหลวงและผึ้งพันธุ์

EFFECT OF *Nosema ceranae* EXTRACTED FROM THAI HONEY BEES ON ENERGETIC STRESS OF *Apis dorsata* AND *Apis mellifera*

RUJIRA PONKIT

Burapha University

2024

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รุจิรา ผลกิจ

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EFFECT OF *Nosema ceranae* EXTRACTED FROM THAI HONEY BEES ON ENERGETIC STRESS OF *Apis dorsata* AND *Apis mellifera*

RUJIRA PONKIT

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The Dissertation of Rujira Ponkit has been approved by the examining committee to be partial fulfillment of the requirements for the Doctor Degree of Philosophy in Biological Sciences of Burapha University

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RUJIRA PONKIT : EFFECT OF *NOSEMA CERANAE* EXTRACTED FROM THAI HONEY BEES ON ENERGETIC STRESS OF *APIS DORSATA* AND *APIS MELLIFERA*. ADVISORY COMMITTEE: GUNTIMA SUWANNAPONG, Ph.D. ROBERT JOHN PAXTON, Ph.D. 2024.

Nosema ceranae is a microsporidian causing nosemosis in all *Apis* species. The ingested spores germinate and proliferate in gut epithelium causing detrimental impacts and inducing mortality of honey bees. The virulence of *N. ceranae* on Thai honey bees especially, *A. dorsata*, is not well understood. This study aimed to investigate the virulence of *N. ceranae* isolated from four Thai honey bee species on *A. dorsata* workers. The impact of *N. ceranae* isolated from *A. mellifera* on energetic stress of *A. dorsata* was then studied. Finally, alternative treatments using stingless bee propolis (*Tretigona apicalis*) and chito-oligosaccharide (COS) were tested to suppress the infection and promote honey bee health. The 100% infection was found when dosed with *N. ceranae* spores isolated from *A. dorsata*, *A. florea*, and *A. mellifera* at 5×10^5 spores per bee with the lowest survival was found in bees dosed with *N. ceranae* from *A. mellifera*. Moreover, *N. ceranae* from *A. mellifera* induced energetic stresses in *A. dorsata* workers by reducing trehalose levels, hypopharyngeal gland protein contents, and midgut proteolytic enzyme activities. Treating with 50% propolis extract and 0.5 ppm COS significantly reduced infectivity in *A. dorsata* and *A. mellifera* workers ($p < 0.0001$). Moreover, propolis and COS improved honey bee health by increasing trehalose levels, protein contents, and proteolytic enzyme activity in infected *bees.* The expression of apidaecin and hymenoptaecin increased in *N. ceranae*-infected *A. dorsata* and *A. mellifera* after treating with propolis, and only hymenoptaecin *in A. mellifera* increased after treating with COS. These results demonstrate that propolis extract can be used as an alternative antibiotic to control *N. ceranae* and improve honey bee health.

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Rujira Ponkit

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CHAPTER 1
INTRODUCTION

1.1 Statements and significance of the problems

Honey bees are well-known as importance pollinators of various agricultural crops and wild flora. They are also known as the producers of honey and other beneficial products for humans (Khalifa et al., 2021; Singh & Takhellambam, 2021; Stein et al., 2017; Suwannapong, 2019). For this importance, the incidence of honey bee population decline in many countries was considered to be a priority. There are many factors contribute to honey bee decline such as chemical spraying, habitat degradation, honey bee pests and diseases including *Nosema* disease (Hadjur, Ammar, & Lefèvre, 2022).

Nosemosis or *Nosema* disease has been suggested as one of factors that might contributing to the weakening and death in honey bees (Galajda, Valencakova, Sucik, & Kandracova, 2021; Hristov, Shumkova, Palova, & Neov, 2020). This disease caused by three microsporidia of genus *Nosema* which are *N. apis*, *N. ceranae* and *N. neumanni*. Recently, they were reclassified into genus *Vairimorpha* (Tokarev et al., 2020), however, it was retained the name *Nosema* here. *N. apis* has been known for more than one hundred years ago as *Apis mellifera* infecting parasite while *N. ceranae* was first discovered in *A. cerana* in China (Fries, Feng, da Silva, Slemenda, & Pieniazek, 1996). Another species, *N. neumanni* was discovered in *A. mellifera* of Uganda (Chemurot, De Smet, Brunain, De Rycke, & de Graaf, 2017). Nowadays, *N. ceranae* has spread worldwide after being transmitted from its original host, *A. cerana* to a new host, *A. mellifera*. Then, in many regions of the world, *N. ceranae* can be found as a natural infection of *A. mellifera* and other honey bee species including the red dwarf honey bee, *A. florea* and the giant honey bee, *A. dorsata* (Chaimanee, Warrit, & Chantawannakul, 2010; Klee et al., 2007; Martin-Hernandez et al., 2018; Suwannapong, Yemor, Boonpakdee, & Benbow, 2011). This distribution could be due to their shared foraging environment (MacInnis, Keddie, & Pernal, 2021; Suwannapong, 2019).

Nosema infection occurs mostly through ingestion of spores when they are eating contaminated food or when they are cleaning up fecal material from infected bees. The spores geminate within the midgut and release polar tubes that transfer their sporoplasm into midgut epithelial cells (Chen, Evans, Smith, & Pettis, 2008; Chen et al., 2009; MacInnis et al., 2021). They draw nutrient from their hosts to grow and multiply inside the ventricular epithelial cells, this causes an energetic stress for honey bees, lower levels of protein content synthesized and secreted from the hypopharyngeal glands (MacInnis, Luong, & Pernal, 2023; Naree, Benbow, Suwannapong, & Ellis, 2021a; Ponkit, Naree, Mayack, & Suwannapong, 2021). Moreover, infection by *Nosema* also causes digestive disorders, behavioral fever, hormonal disturbance, immunity depletion and decrease in the honey bee lifespan resulting in decreasing bee population, affecting the numbers of the most important insect pollinators (Chaimanee & Chantawannakul, 2016; Dussaubat et al., 2013; Galajda et al., 2021; Goblirsch, Huang, & Spivak, 2013; MacInnis et al., 2023). *N. ceranae* also alter the expression of the honey bee midgut proteome to create an environment favorable for parasite development (Kurze et al., 2016; Vidau et al., 2014).

Many compounds have been used to control *Nosema* such as fumagillin. It is the antibiotic isolated from the fungus *Aspergillus fumigatus* that approved for control of *Nosema* disease in honey bees and has been extensively used in United States apiculture. Treatment of an antibiotic fumagillin can reduce *Nosema* spore counts, but it is possible for the disease to recur (Giacobino et al., 2016; Huang, Solter, Yau, & Imai, 2013; Maistrello et al., 2008), and have toxicity for honey bee and human (El-Seedi et al., 2022; Giacobino et al., 2016; Huang et al., 2013; van den Heever et al., 2015). Therefore, many countries have banned fumagillin for agricultural used. A study suggested that fumagillin is less efficient in controlling *N. ceranae* (Binganski et al., 2024). For resolve those concerns, numerous natural compounds are being employed as antibiotics for killing *Nosema* without creating adverse effects on adult honey bees, such as thymol, vetiver essential oil, lysozyme, resveratrol and several bioactive compounds from plant extracts (Chaimanee et al., 2021; Chen et al., 2021; Garrido et al., 2024; Maistrello et al., 2008; Porrini et al., 2011). The product from bees such as propolis is the one of popular product used as antibiotic. Propolis from

honey bee, *A. mellilfera* and stingless bee, *Tetrigona apicalis* are also high efficiency for control *Nosema*. The chemical composition of propolis, mainly phenolic acid, flavonoid and its derivatives, might have toxic effects on *N. ceranae* spores, cause abnormal structure of *N. ceranae* spores resulting in interfering or inhibiting spore growth and development (Naree, Ellis, Benbow, & Suwannapong, 2021b; Suwannapong, Maksong, Phainchajoen, Benbow, & Mayack, 2018; Yemor, Phiancharoen, Benbow, & Suwannapong, 2016). Furthermore, treating with propolis also improved honey bee health which was suffered from *Nosema* infection (Naree, Ponkit, Chotiaroonrat, Mayack, & Suwannapong, 2021c; Ponkit et al., 2022). Another natural compound in particular chito-oligosaccharides (COS), the chemical containing antimicrobial activity and cell healing was also evaluated for the control of *Nosema* disease and enhancing the immunity in honey bees (Borges, Guzman-Novoa, & Goodwin, 2020; Naree et al., 2021c; Ponkit et al., 2022; Saltykova et al., 2016; Valizadeh, Guzman-Novoa, Petukhova, & Goodwin, 2021).

When honey bees get infection by pathogen, they have evolved both individual and group social immunity to defend against pathogens, pests and parasite (DeGrandi-Hoffman & Chen, 2015). They can defend themselves by activating their immune systems (Negri et al., 2017; Sinpoo, Paxton, Disayathanoowat, Krongdang, & Chantawannakul, 2018) which possesses by four immune pathways: Toll, Imd, JNK, and JAK/STAT (Chaimanee & Chantawannakul, 2016; Evans et al., 2006; Pluta & Sokol, 2020). Humoral immunity is one of honey bee defense mechanisms that involves the synthesis of antimicrobial peptides (AMPs) against bacteria, fungi, parasites and viruses. The AMPs such as abaecin, apidaecin, hymenoptaecin, and defensin were found in *A. mellifera* which have a wide-range of activity against microorganism (Evans et al., 2006; Li, Ma, & Jiang, 2022; Roy et al., 2023). Moreover, those AMPs also found in other honey bee species including *A. cerana*, *A. florea*, and *A. dorsata* (Chaimanee et al., 2013; Sinpoo et al, 2018).

According to *A. cerana* foragers, hosts of *N. ceranae,* have overlapping foraging area with other native honey bee species of Thailand including *A. dorsata*, this behavior may cause the new host, as *A. dorsata* might get *N. ceranae* infection by contracting with the contaminated food from foraging areas (Suwannapong et al., 2018). Several studies have been conducted to investigate the ability of *N. ceranae*

different isolates to infect honey bees. Previous research has revealed that *N. ceranae* isolates from different geographical regions varied in their capacity to infect honey bees (Chaimanee et al., 2013). One study also found that *N. ceranae* isolated from different hosts, *A. cerana* and *A. mellifera*, has differences in their ability to infect honey bees (Chaimanee & Chantawannakul, 2016). This could be due to differences in host responses or the virulence levels among *N. ceranae* isolates. However, it is still unclear if different *N. ceranae* isolates from different honey bee species in Thailand are similarly virulent in the giant honey bee, *A. dorsata*.

This study aims to investigate the virulence of *N. ceranae* isolated from four different hosts *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* on the worker of giant honey bees, *A. dorsata* which assume as a new host. The infective ability of different *N. ceranae* isolates was compared by measuring the infectivity, infection rate, and mortality of worker bees using cage experiments. Moreover, the effect of *N. ceranae* infection on honey bee health was also studied by measuring the trehalose levels in bee hemolymph, the protein content in hypopharyngeal glands, and the total midgut proteolytic enzyme activity of the worker bees. Then, the natural compounds such as propolis extract from stingless bees, *Tetrigona apicalis* and other natural products in particular chito-oligosaccharides (COS), the chemical containing antimicrobial activity and cell healing were evaluated for the control of *Nosema* disease, enhancing the bee immunity, and improving honey bee health. (Borges et al., 2020; Naree et al., 2021c; Ponkit et al., 2022; Saltykova et al., 2016; Suwannapong et al., 2018; Valizadeh et al., 2021). Those natural compounds also were treated in *A. mellifera* workers which is susceptible to *N. ceranae* infection (Chaimanee et al., 2013).

1.2 Objectives

1.2.1 To examine the virulence of *Nosema ceranae* isolated from four honey bee species of Thailand; *Apis cerana*, *A. dorsata*, *A. florea*, and *A.mellifera* in the giant honey bee, *A. dorsata* workers.

1.2.2 To investigate the effect of *N. ceranae* isolated from *A. mellifera* on energetic stress of *A. dorsata* workers using a dosage that results in 100% infection (ID_{100}).

1.2.3 To investigate the impact of natural products; propolis extract of stingless bee, *Tetrigona apicalis* and chito-oligosaccharide (COS), for the control of *N. ceranae* infection in *A. dorsata* workers compared to those of *A. mellifera* workers.

1.2.4 To measure the expression of immune-related genes of *A. dorsata* and *A. mellifera* workers response to the infection of *N. ceranae,* and after treating with propolis and COS.

1.3 Hypotheses

1.3.1 Hypothesis I

H_0: The virulence of *Nosema ceranae*, isolated from *Apis cerana, A. dorsata, A. florea*, and *A. mellifera*, infected workers of *A. dorsata* will not differ from those of controls.

H_1: The virulence of *N. ceranae*, isolated from *A. cerana, A. dorsata, A. florea*, and *A. mellifera*, infected workers of *A. dorsata* will differ from those of controls.

1.3.2 Hypothesis II

H_0: The mortality of *N. ceranae*, isolated from *A. mellifera*, infected workers of *A. dorsata* under the ID_{100} dosage will not differ from those of controls.

H_1: The mortality of *N. ceranae*, isolated from *A. mellifera*, infected workers of *A. dorsata* under the ID_{100} dosage will differ from those of controls.

1.3.3 Hypothesis III

H_0: The infectivity and the infection ratio of *N. ceranae*, isolated from *A. mellifera*, infected workers of *A. dorsata* under the ID_{100} dosage will not differ from those of controls.

H_1: The infectivity and the infection ratio of *N. ceranae*, isolated from *A. mellifera*, infected workers of *A. dorsata* under the ID_{100} dosage will differ from those of controls.

1.3.4 Hypothesis IV

H_0: Trehalose level in hemolymph of *N. ceranae,* isolated from *A. mellifera*, infected workers of *A. dorsata* under the ID_{100} dosage will not differ from those of controls.

H_1: Trehalose level in hemolymph of *N. ceranae*, isolated from *A. mellifera*, infected workers of *A. dorsata* under the ID_{100} dosage will differ from those of controls.

1.3.5 Hypothesis V

H_0: Protease activity in ventricular epithelium of *N. ceranae,* isolated from *A. mellifera*, infected workers of *A. dorsata* under the ID_{100} dosage will not differ from those of controls.

H_1: Protease activity in ventricular epithelium of *N. ceranae,* isolated from *A. mellifera*, infected workers of *A. dorsata* under the ID_{100} dosage will differ from those of controls.

1.3.6 Hypothesis VI

H_0: The mortality, infectivity, and infection ratio of *N. ceranae* isolated from *A. mellifera*, infected workers of giant honey bee, *A. dorsata* compared to European honey bee, *A. mellfiera* after treated with propolis extract from *Tetrigona apicalis* will not differ.

H_1: The mortality, infectivity, and infection ratio of *N. ceranae* isolated from *A. mellifera*, infected workers of giant honey bee, *A. dorsata* compared to European honey bee, *A. mellfiera* after treated with propolis extract from *T. apicalis* will differ.

1.3.7 Hypothesis VII

H_0: The mortality, infectivity, and infection ratio of *N. ceranae* isolated from *A. mellifera*, infected workers of giant honey bee, *A. dorsata* compared to European honey bee, *A. mellfiera* after treated with COS will not differ.

H_1: The mortality, infectivity, and infection ratio of *N. ceranae* isolated from *A. mellifera*, infected workers of giant honey bee, *A. dorsata* compared to European honey bee, *A. mellfiera* after treated with COS will differ.

1.3.8 Hypothesis VIII

H_0: The expression of immune-related genes of *N. ceranae*-infected *A. dorsata* and *A. mellifera* after treated with propolis extract from *T. apicalis* or COS will not differ from those of controls.

H_1: The expression of immune-related genes of *N. ceranae*-infected *A. dorsata* and *A. mellifera* after treated with propolis extract from *T. apicalis* or COS will differ from those of controls.

1.4 Contribution to knowledge

1. To understand the virulence of *Nosema ceranae* spores isolated from *Apis cerana, A. dorsata, A. florea* and *A. mellifera* workers infected the workers of *A. dorsata*.

2. Understanding of infection of *N. ceranae* in *A. dorsata* will also help in disease control program.

3. The studies will be provided information for control *Nosema* disease in honey bee colonies such as screening of natural products.

4. To understand how honey bee immunity responds to *N. ceranae* infections.

1.5 Scope of the study

The virulent of *Nosema ceranae* spores isolated from different species of Thai honey bees; *Apis cerana, A. dorsata, A. florea* and *A. mellifera* on giant honey bee, *A. dorsata* workers was studied by measuring the mortality, infectivity and infection rate. The effect of *N. ceranae* isolated from *A. mellifera* on energetic stress of *A. dorsata* workers was investigated by measuring the trehalose level in bee's hemolymph, the hypopharyngeal gland protein content, and the midgut proteolytic enzyme activity of *N. ceranae*-infected bees and control bees. The effect of natural products such as propolis extract from stingless bee, *Tetrigona apicalis*, and chito-oligosaccharide (COS) on *Nosema* infection in *A. dorsata* and *A. mellifera* workers were examine mortality, infectivity, infection rate, infection ratio, trehalose level in hemolymph, protein contents of hypopharyngeal glands, and the proteolytic enzyme

activity. The expression of immune genes of *N. ceranae*-infected bees were also studied. Newly emerged *A. dorsata* or *A. mellifera* workers were caged in groups of 50 bees and kept in an incubator at 34 ± 2 °C with relative humidity between 50 - 55%. Three replicates of each were used. Each cage was fitted with 2 gravity feeders, one containing water and another containing 50% w/v sucrose solution which were replenished every 2 days during the experiment. Sufficient food was prepared by 60 g pollen mixed with 17 ml of 50% sucrose solution (w/v) to feed the honey bees.

CHAPTER 2
LITERATURE REVIEWS

2.1 The important of honey bees

Honey bee is the most important insect involved in agriculture and food production. They are the most effective pollinators for both wild and crop plants, providing valuable pollination services for a variety of agricultural crops. An estimated 34% of global agricultural pollination services are attributed to the managed European honey bee, *Apis mellifera,* and it contributed more than 10 billion dollars to agriculture in the United States and more than a thousand million Bath in Thailand (Breeze, Bailey, Balcombe, & Potts, 2011; Calderone, 2012; Suwannapong, 2019).

Honey bee forage and collect nectar and pollen grains from flowers for their nutrition, which causes pollination to occur. Pollination process by pollen grains transfer from the male anther of a flower to the female stigma, either within the same flower (self-pollination) or between plants (cross-pollination), which is necessary for plant reproduction. Honey bee pollination can improve quantity and quality of fruits and crops (Brewer, Miwa, & Hanford, 2023; Wu et al., 2021), and it involve in one-third of human food supply. Many crops were pollinated by honey bees such as cucumber (*Cucumis sativus* Linn), cranberry (*Vaccinium oxycoccos* Linn), pear (*Pyrus communis* Linn), guava (*Psidium guajava* Linn), coconut (*Cocos nucifera* Linn), citrus (*Citrus* spp.), sesame (*Sesamum indicum* Linn), oilseed rape (*Brassica napus* Linn), buckwheat (*Fagopyrum esculentum* Moench), apple (*Malus domestica* Borkh), sunflower (*Helianthus annuus* Linn) (Brewer et al., 2023; Khalifa et al., 2021). In Thailand, more than a hundred plant species were pollinated by honey bees such as longan (*Dimocarpus longan*), magosteen (*Garcinia mangstana*) litchi (*Lychee chinensis*), durian (*Durio zibethinus* Murray), rambutan (*Nephelium lappaceum*), opal basil (*Ocimum basillicum* L.), jasmine rice (*Oryza sativa* L.), soybean (*Glycine* max), plum (*Prunus mume* Sieb.), sedge (*Schoenoplectus juncoides* (Roxb.), corn (*Zeamays*) (Suwannapong, 2019; Suwannapong, Benbow, & Nieh, 2011). Some plants species depend on honey bee pollination. It means that if without honey bee, it will not produce fruit.

Honey bee also produce the valuable products from their hive such as honey, pollen, propolis, beeswax, royal jelly and venom, which benefit to humans. These products had anti-imflammatory, anti-bacterial, anti-fungal, anti-viral and antioxidant activities so it was also used as medicine (Sforcin, Bankova, & Kuropatnicki, 2017).

2.2 Honey bee Biology

Honey bees are members of the order Hymenoptera, family Apidae, and genus *Apis*, which comprise 11 recognized species. They are classified into three distinct groups which were: cavity-nesting bees (*A. mellifera*, *A. cerana*, *A. koschevnikovi*, and *A. nulensis*), giant bees (*A. dorsata*, *A. laboriosa*, *A. binghami*, *A. nigrocincta*, and *A. breviligula*), and dwarf bees (*A. florea* and *A. andreniformis*) (Hepburn & Radloff, 2011; Lo, Gloag, Anderson, & Oldroyd, 2010; Suwannapong, 2019). In Thailand, there are four native honey bee species which are *A. florea, A. andreniformis*, *A. cerana*, *A. dorsata, A. laboriosa*, and one exotic species which is *A. mellifera* (Suwannapong et al., 2011a; Voraphab et al., 2024). They are classified into two groups based on their nest. The first group open-air nesting honey bees: *A. dorsata, A. laboriosa, A. florea* and *A. andreniformis*, the another group is multiple combs gravity-nesting honey bees: *A. mellifera* and *A. cerana* (Kitnya et al., 2020; Suwannapong, 2019; Suwannapong et al., 2011a). The taxonomy of honey bees in Thailand are classified as follows:

Kingdom Animalia
Phylum Arthropoda
Class Insecta
Order Hymenoptera
Family Apidae
Genus *Apis*
Species *A. andreniformis* Smith, 1858
A. cerana Fabricius, 1793
A. dorsata Fabricius, 1793
A. florea Fabricius, 1787
A. mellifera Linnaeus, 1758 (Ruttner, 1988)

A. laboriosa, Smith, 1871 (Sakagami, Matsumura, & Ito, 1980)

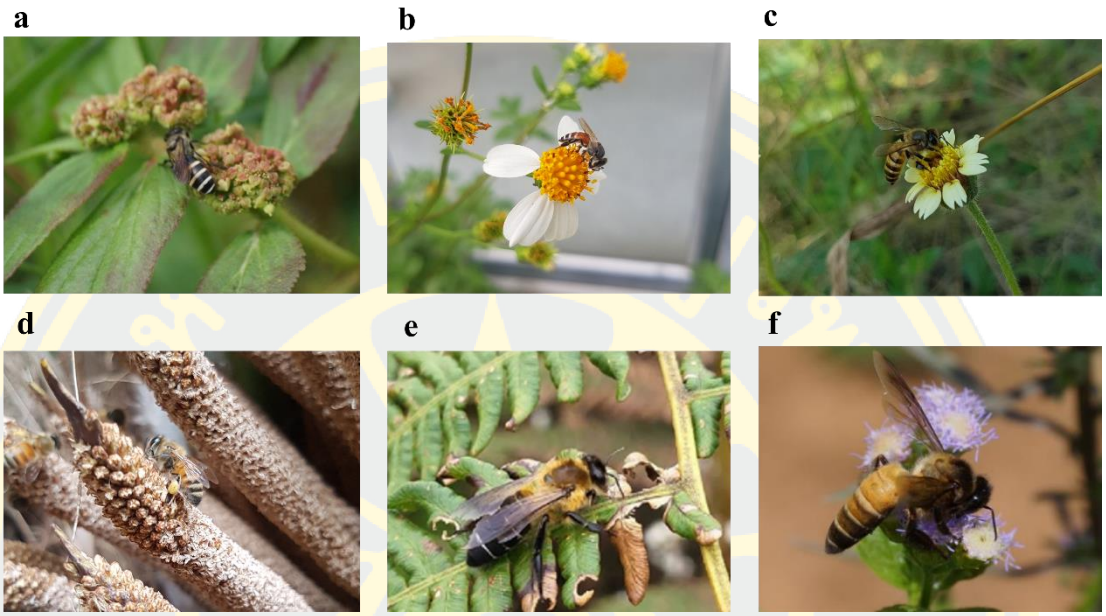

Figure 2.1 Honey bees in Thailand; *A. andreniformis* (Suwannapong, 2019) (a), *A. florea* (b), *A. cerana* (c), *A. mellifera* (d), *A. laboriosa* (e) (Voraphab et al., 2024), and *A. dorsata* (f) (Suwannapong, 2019).

Honey bees are highly eusocial insects, which means that they live together in large, well-organized and sophisticated communities known as colonies. Each honey bee colony typically consists of three different type of individuals known as castes: a queen, drones and workers. All honey bee castes pass through the same four developmental stages: egg, larva, pupa and adult and each caste has its own function in the colony (Wojciechowski et al., 2018; Yadav, Kumar, & Jat, 2017).

Queen is effectively the mother of the colony. She is the only female that is completely sexual development. In general, there is only one queen in each colony and she can live several years under healthy conditions. The queen is responsible for laying eggs for a colony. She lays both fertilized eggs (females) and unfertilized eggs (males), and she controls many colony activities by her production of pheromones. The abdomen of queen is much larger than drone and worker but her wings have shortest.

Drones are male bees that develop from unfertilized eggs. There are a few hundred or thousand presents in the colony during the mating season. They do not work in the colony and die after workers stop feeding them. The only function of drone is to mate with virgin queen, and they die in the process. The body sizes of drones are larger than workers, though usually smaller than the queen bee. They also have larger eyes when compare with worker and queen bees.

The workers are female bee that develop from fertilized eggs laid by the queen. They do almost all colony tasks such as brood rearing, comb building, house cleansing, foraging, and colony defensing. Their tasks among worker bees depend primarily on their ages. Workers are smaller than the queen and drones, but there have the highest number in the colony. There are about 8,000-25,000 workers in *A. andreniformis* and *A. florea* colonies, 40,000-50,000 workers in *A. mellifera* colony, 20,000-40,000 workers in *A. cerana* colony and 50,000-80,000 in *A. dorsata* colony (Suwannapong et al., 2011a).

2.3 The cavity nesting honey bee species

Cavity nesting honey bees generally build their nests as multiple parallel combs inside cavices or hollows in trees. Combs are attached to the walls along their tops and sides (Shanas, Anju, & Mashhoor, 2022). There are two species of cavity nesting honey bees in Thailand, which are *Apis cerana* and *A. mellifera*.

2.3.1 The Eastern honey bee, *A. cerana* Fabricius, 1793

Apis cerana is the Asiatic or the oriental honey bee because they are only found in Asia, from Iran in the east to Pakistan in the west, from Japan in the north to the Philippines in the south, and they are found throughout Thailand. This species does not live only in tropical and subtropical areas of Asia, but also in colder areas as Siberia, Northern china and the high mountain area of the Himalayan region (Gupta, 2014).

Apis cerana is in the same subgenus as the European honey bee, *A. mellifera*. They are genetically and morphologically subdivided into several subspecies that differ in their ecology and behavior. The body size of *A. cerana* workers are generally smaller than *A. mellifera*. However, the morphological variation

has been found within both *A. cerana* and *A. mellifera*, such as workers of the southern subspecies of *A. cerana* are smaller than *A. mellifera* workers, while workers of northern subspecies of *A. cerana* are larger than some of the African subspecies of *A. mellifera* (Koetz, 2013).

Figure 2.2 The Eastern honey bee, *A. cerana* forager.

Apis cerana generally builds multiple combs arrange parallel to one another in the dark cavities and crevices, like hollow tree trunks. The normal nesting site is usually close to the ground, not more than 4-5 meters high. *A. cerana* are managed for honey production and crop pollination. Their honey yield is less than *A. mellifera* colony but their beeswax is used to treat and heal wounds. Moreover, they are also strongly resistant to *Varroa*, a serious pest of the European honey bee (Suwannapong et al., 2011a; Wongsiri et al., 1996).

Figure 2.3 The multiple combs nesting of *A. cerana*

2.3.1 The Western honey bee, *A. mellifera* Linnaeus, 1758

Apis mellifera normally builds multiple comb nests in the cavities like *A. cerana*. Their body size are slightly larger than the Asiatic honey bee, *A. cerana*. This species is the primary species maintained by the beekeepers for both honey production and pollination. They are the most frequent floral visitor in natural habitats and crop plants worldwide (Hung, Kingston, Albrecht, Holway, & Kohn, 2018). They pollinated many domestic and imported fruits and vegetables, including avocados, soybeans, broccoli, celery, squash, sunflowers, cucumbers, citrus fruit, peaches, kiwis, cherries, cranberries and melons. Some crops such as almonds need 100% honey bee for pollination (Khalifa et al., 2021; Papa et al., 2022).

Apis mellifera is native to Africa, Europe and the Middle East. Humans have introduced *A. mellifera* to other continents since the 17th century, and now they are found all around the world, including East Asia, Australia and North and South America. They were brought to Thailand for beekeeping and used for honey production and pollination of longan, litchi, Siam weed and other crops (Chama, 2016; Han, Wallberg, & Webster, 2012; Suwannapong et al., 2011a).

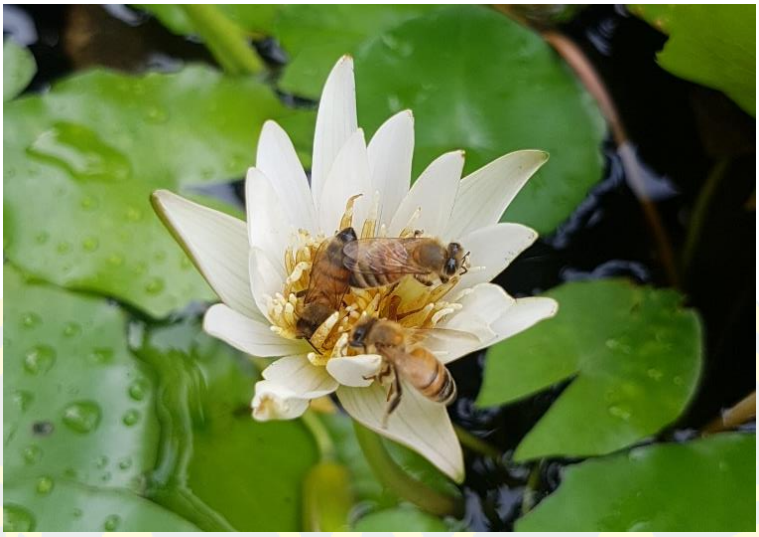

Figure 2.4 The Western honey bee, *A. mellifera* foragers forage on water lily flower.

Figure 2.5 *Apis mellifera* hives of agricultural technology promotion center on economic insects, Chiang Mai Province, Thailand.

2.4 The open-air nesting honey bee species

The open-air nesting honey bee usually build a single comb nest attached to a tree branch, rooftops and cliff-sides. There are three species of open-air nesting honey bee in Thailand which are *Apis andreniformis*, *A. florea*, and *A. dorsata*.

2.4.1 The black dwarf honey bee, *A. andreniformis* Smith, 1858

Apis andreniformis is a relatively rare species of honey bee. They are recently separated from *A. florea* since there are sites where both *A. andreniformis* and *A. florea* live co-specifically. The body size of *A. andreniformis* is clearly smaller and more slender than *A. florea* and their abdomen has a dark color overall, with distinct light bands. *A. andreniformis* is distributed throughout tropical and subtropical Asia, including Southern China, India, Burma, Laos, Vietnam, Malaysia, Indonesia and the Philippines (Palawan) (Gupta, 2014; Suwannapong et al., 2011a; Wongsiri et al., 1996). This species was reported in Thailand by Wongsiri et al. (1990), who found *A. andreniformis* distribute from the coastal flats and near the foothill areas (1–100 meters above sea level) of Chanthaburi province to high mountainous and forest areas at about 1600 meters altitude in the northern parts of Thailand (Wongsiri et al., 1996).

Figure 2.6 The black dwarf honey bee, *A. andreniformis* forager (Suwannapong, 2019).

The nests of *A. andreniformis* are made of a single, exposed comb under the small twigs between 1 and 15 meters far from the ground, although the average altitude is 2.5 meters. They generally nest in quiet forests and darker areas where there is 25 to 30% of normal sunlight. They are commonly found hanging in small trees, shrubs or bushes and usually hidden behind leaves or branches to avoid

detection. When the nest is disturbed, a tail-shaped group of bees will appear at the bottom of the nest for prepare to attack an intruder. This tail will disappear as the bees in it take to the air and engage in nest defense (Suwannapong et al., 2011a; Wongsiri et al., 1996).

Figure 2.7 A single comb nest of *A. andreniformis* in Chantha Buri Province, Thailand (Suwannapong, 2019).

2.4.2 The red dwarf honey bee, *A. florea* Fabricius, 1787

The red dwarf honey bee, *A. florea*, is one of two species of small honey bees. A worker is normally 7–10 mm in body length that somewhat similar to *A. andreniformis* in appearance. *A. florea* has a distinctly reddish abdomen and has light whitish hairs covering its thorax and legs, while the hairs of *A. andreniformis* are dark. *A. florea* build exposed nest with a single comb on a single branch (Suwannapong et al., 2011a). The distribution area of *A. florea* is generally confined to warm climates. This species is present in the warmer parts of Oman, Iran and Pakistan throughout the Indian sub-continent and Sri Lanka. It is found as Far East as Indonesia, but its primary distribution center is Southeast Asia. It rarely found at altitudes above 1,500 meters and not found in north of the Himalayas. *A. florea* are frequently found in tropical forests, in woods and even in farming areas. It has a much wider distribution than its sister species, *A. andreniformis* (Gupta, 2014; Wongsiri et al., 1996).

Figure 2.8 The red dwarf honey bee, *A. florea* foragers forage on a water lily flower.

Figure 2.9 A single comb nest of *A. florea* in Samut Songkhram Province, Thailand.

Apis florea can survive in very hot and dry climates with ambient temperatures reaching 50 °C or more. Worker bees live longer than those of other *Apis* species, usually about 60 days. This species is generally distributed throughout Thailand and it is an economically important species for crop and wild plant

pollination. They can fly approximately 750 meters far from the nest for foraging and can produce honey up to 4 kg in each nest (Wongsiri et al., 1996).

2.4.3 The Himalayan giant honey bee, *A. laboriosa* Smith, 1980

Apis laboriosa was classified as a full species (Sakagami et al., 1980) after previously being classified as subspecies of *A. dorsata*. Recently, genetic and morphological analysis has confirmed that *A. laboriosa* from several regions of Arunachal Pradesh, India is distinct from *A. dorsata* (Kitnya, Otis, Chakravorty, Smith, & Brockmann, 2022). *A. laboriosa* is slightly bigger than its sister species, *A. dorsata*. Their body length is approximately 3 cm. Its thoracic hair colour is tawny yellow which is different from *A. dorsata*. The first two tergites are black or grey in callow in adult bees (Kitnya et al., 2020; Sakagami et al., 1980). It is a largest species of genus *Apis* and usually build a single comb nest that almost always hangs from rock cliffs at high altitudes from 230 - 4270 m above sea level (Kitnya et al., 2020). They produced a lot of honey accounts for 70-80% of the honey production in India and Napal (Woyke, Wilde, & Wilde, 2012).

Their distribution is primarily found in the Hindu Kush Himalayan region of southern Asia, and also found in the mountainous regions of Bhutan, China, India, Laos, Myanmar, Nepal, and Vietnam (Kitnya et al., 2020). Moreover, first discovery of *A. laboriosa* in Thailand was published in 2024 (Voraphab et al., 2024). This bee species was found in northern Thailand, Doi Pha Hom Pok National Park, Chaing Mai Province. The specimens of *A. laboriosa* collected from northern Thailand has 15.46 ± 0.65 mm. in body length which is larger than *A. dorsata,* and the authors also suggest that *A. laboriosa* might be occur in other highland regions of northern Thailand (Voraphab et al., 2024).

Figure 2.10 The Himalayan giant honey bee, *A. laboriosa* forager (Voraphab et al., 2024).

Figure 2.11 The single comb nests of *A. laboriosa* at Doi Pha Hom Pok National Park, Chaing Mai Province, Thailand (Voraphab et al., 2024).

2.4.4 The giant honey bee, *A. dorsata* Fabricius, 1793

The giant honey bee, *A. dorsata* has a widespread distribution throughout South and Southern Asia and also in Thailand (Wongsiri et al., 2000). They have the largest in body size (17–20 mm. long) that allows these bees to have a greater flight and foraging range than those of other honey bee species. This species builds a single, exposed comb that hang freely from the tree branches or cliffs and the building structures, normally from 3 to 25 meters far from the ground. The nests of *A. dorsata* are mostly semi-circular in shape and vary in size, with dimensions of 1.5 x 1

meters. In general, nest of *A. dorsata* may occur singly or several nests that are formed aggregately in group of 20-100 nests on a single tree or on a cliff site (Suwannapong et al., 2011a; Tan, 2007; Weihmann, Waddoup, Hotzl, & Kastberger, 2014; Wongsiri et al., 2000).

Figure 2.12 A single comb nest of giant honey bee, *A. dorsata* in Samut Songkhram Province, Thailand.

Apis dorsata prefer smooth surfaces over unevenly grooved surfaces for nesting. They made nest in different directions and on various inclined supports. Most of the colonies nested on supports with an inclination from 0˚ to 45˚, and inclination of nesting support with 15˚ to 30˚ seemed to be maximally preferred. The direction of *A. dorsata* nests were built according to the wind currents. Sihag (2017) reported that about 48.6% of *A. dorsata* nests were built in an East-West direction in the North-western region of India, followed by 29.4% in a northeast-southwest direction, because western or northern winds flow in this region and the majority of the nests were built parallel to these currents. In addition, *A. dorsata* colonies migrate seasonally and they appear to return to their previously occupied sites. The seasonal migration in this honey bee species vary from place to place depending on the local climatic and floral conditions (Robinson, 2012).

The structure of *A. dorsata* nest is comprised of hexagonal cells and horizontal in orientation. The cells used for rearing drone and workers brood are the same size, but the capping of the drone cells is domed much higher than worker cells. Honey is usually stored in the highest part of the comb. Next to the honey is a region of stored pollen, and the rest of the comb contains brood. Both the diameter and depth of honey storage cells are greater than the cells use to rear brood (Buawangpong, Saraithong, Khongphinitbunjong, Chantawannakul, & Burgett, 2014; Tan, 2007).

The nest of *A. dorsata* is covered on both sides with 'bee curtain', which consists of several layers of densely clustered colony members hanging loosely attached on each other (Weihmann et al., 2014). This curtain enables brood incubation within narrow temperature limits at 35 °C and protects the nest during storms with heavy wind and rain (Kastberger, Waddoup, Weihmann, & Hoetzl, 2016). The bee curtain has different functional regions that consist of the quiescent zone, where the bees hang seemingly motionless, with their heads upwards and the abdomens downwards. Outside of this zone are the rim areas and the attachment zone (the zone of bees closes to the tree branch) and the mouth zone, where the foraging bees depart and arrive and where communication dances by scouts announcing the discovery of food sources take place. This zone is established at the sunny side of the nest (Kastberger et al., 2012; Weihmann et al., 2014).

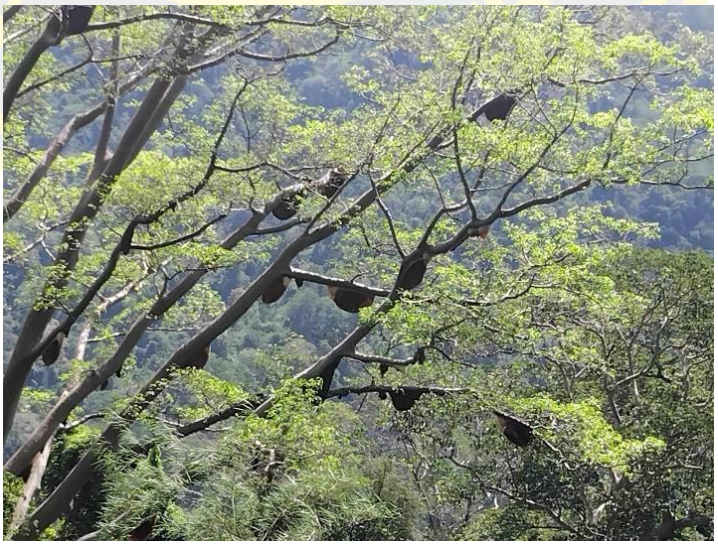

Figure 2.13 The aggregated single open nesting of *A. dorsata*, Khao Yai National Park, Nakhon Ratchasima Province, Thailand.

According to *A. dorsata* build the exposed nest, they are directly exposed to a variety of predators such as wasps, hornets and birds. Defense strategies in giant honey bee may involve physical contact with aggressors. When they face attacks from wasps, they have developed specific defense behaviors such as 'heat balling' in which they heat their thoraces by their flight muscles to above 45 °C which is dangerous to wasps (Kastberger, Schmelzer, & Kranner, 2008). Honey bee colonies also defend themselves without physical contact such as the shimmering behaviour. This behaviour involves a display of social waves that bee curtain flip their abdomens upwards in a wave-like visual pattern to repel aggressor from their nest (Koeniger, Kurze, Phiancharoen, & Koeniger, 2017).

Apis dorsata is considered to be extremely important to both honey and agricultural producers in southern Asia. Many crops throughout southern Asia depend upon *A. dorsata* pollination such as cotton, mango, coconut, coffee, pepper, star fruit and macadamia (Suwannapong et al., 2011a). In Pakistan, they pollinated 61 crops, which include 26 fruit crops, 7 oilseed, 4 pulses, 19 vegetables, 2 spices and 3 nut trees (Irshad & Stephen, 2014), and they also role in the pollination of wild flora or in the forest plant species. Moreover, *A. dorsata* is highly efficient in exploiting floral rewards. Its flight range from their hive is very large and tendency to visit multiple trees or flowers. *A. dorsata* can forage during moonlit nights (Raju & Rao, 2004). Therefore, they are the only social bee that foraged on several dipterocarp tree species at Lambir, Sarawak in which the flowers open before sunrise (05:00– 06:00 a.m.) and after sunset (06:00–08:00 p.m.) (Momose et al., 1998). Moreover, it appears that *A. dorsata* is the only pollinator of the introduced plant *Ruellia simplex*. This plant is a species of flowering plant that native of Mexoco, the Caribbean and South America. It was introduced In Thailand, and grown extensively as a garden plant and for hedges in parks and gardens (Hawkeswood & Sommung, 2016).

In addition, *A. dorsata* are also important as a source of honey in India and some other tropical Asian countries. Locals are mainly interested in harvesting honey, and sometimes wax and brood, from their colonies, because *A. dorsata* combs are so large and such efficient honey producers. They can possess 4 kg of honey after nesting only 3-4 weeks (Tan, 2007), and some of the large colonies can contain up to

45 kilograms of honey that provide an important source of income. In some places commanding the best prices in local markets (Suwannapong et al., 2011a).

However, recent reports revealed that *A. dorsata* colonies tend to decline which might cause by deforestation, disease, climate change, pesticide and parasite (Rattanawannee, Chanchao, Lim, Wongsiri, & Oldroyd, 2013; Sihag, 2014). *N. ceranae* is the one of parasite that cause nosemosis in honey bees. It first discovered in *A. cerana* but has also been found in other *Apis* spp. including *A. dorsata* (Chaimanee et al., 2010; Suwannapong et al., 2011b; Traver & Fell, 2015). This parasite invades the midgut epithelial cells of honey bees and multiply intracellularly, causing cells damage. The production of digestive enzymes and the absorption of food of honey bee is impaired (Soklic & Gregorc, 2016), causing lower energy and shorten lifespan in infected bees that can result in reduced pollen collection and honey yield or even death of the colony. The decline of pollinators can result in loss of pollination services which have impact on ecological and economic. It also affects the maintenance of wild plant diversity, wider ecosystem stability, crop production, food security, and human consumption (Kovács-Hostyánszki, Földesi, Báldi, Endrédi, & Jordán, 2019; Sihag, 2014).

2.5 The hypopharyngeal glands of honey bee

Hypopharyngeal gland is the brood food gland of honey bees. This gland are paired and located in the honey bee's head in front of the brain between the compound (Ahmad, Khan, Khan, & Li, 2020). and delivers its secretion via collecting duct to the hypopharyngx (Klose, Rolke, & Baumann, 2017).

The structure of hypopharyngeal gland is compose of several secretory units or acini which opened into a secretory duct that passed through the mouthparts of honey bee (Ahmad et al., 2020). The acini size is dependent on its activity and honey bee age. Hypopharyngeal glands were inactive in newly immerged bee. It has small in size and it will develop by the age of 3 days. The largest size of acini and highest number of secretory vesicles can be found in honey bee age 6-15 days. After that, acini will decrease in size and secretory activities (Ahmad et al., 2020; Škerl & Gregorc, 2009).

The development of honey bee's hypopharyngeal gland is closely related to honey bee age (Suwannapong, 2007; Suwannapong, Chaiwongwattanakul, & Benbow, 2010). It well developed when bees are nurse and it degenerate when bees become foragers (Brouwers, 2015). In nurse bee, this gland is voluminous and highly produces protein-rich secretions which is fraction of royal jelly used to feed young larvae and queen. When the worker become foraging, their hypopharyngeal gland decrease in size and produce lower of protein. However, their gland produces some enzyme which involved in carbohydrate metabolism such as invertase. This enzyme was used to break down sucrose into fructose and glucose in foragers (Hu et al., 2019; Suwannapong, 2007). However, the degenerate of this gland is flexible and depends on the colony condition such as honey bee diet and the presence of brood in the colony (Hrassnigg & Crailsheim, 1998; Jack, Uppala, Lucas, & Sagili, 2016; Ohashi et al., 2000). Several factors can affect the development of hypopharyngeal glands. Food sources especially protein from pollen is the one of factors that affect the development and activities of the glands (Omar et al., 2016).

Figure 2.14 Light micrograph of hypopharyngeal gland of *A. dorsata* nurse bee.

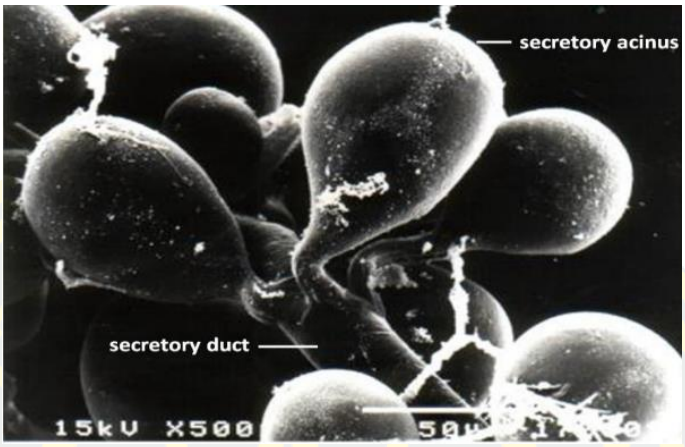

Figure 2.15 Scanning electron micrograph of hypopharyngeal gland of *A. andreniformis* nurse bee (Suwannapong, 2019).

2.6 *Nosema* disease of honey bees

Nosema disease or nosemosis is the one factor that associated with colony collapse disorder or CCD. CCD is characterized by a rapid loss of adult bees from colonies containing a queen, capped brood and food stores, reduce colony strength and heavy winter mortality in the USA and globally (Lee et al., 2015; Seitz et al., 2016). *Nosema* disease is a serious disease of adult honey bees, caused by three species of microsporidia, *N. apis*, *N. ceranae* and *N. neumanni*. Adult worker bees, drones and queens can be infected (Genersch, 2010; Peng, Baer-Imhoof, Millar, & Baer, 2015). However, recent work demonstrated that larvae could be infected by *Nosema* (Eiri, Suwannapong, Endler, & Nieh, 2015; Lee et al., 2015), but spore counts were lower than those typically found in bees infected as adults (BenVau & Nieh, 2017). *N. apis* was first reported over one hundred years ago as a parasite of *A. mellifera* by Zander (1909). After the discovery of *N. apis*, *N. ceranae* was observed in Asian honey bee, *A. cerana* by Fries et al. (1996). However, *N. ceranae* has jumped host from *A. cerana* to *A. mellifera* and it is found in colonies of *A. mellifera* worldwide (Martin-Hernandez et al., 2018). Experimental infection of the European honey bee, *A. mellifera* with *N. ceranae* showed higher spore loads and mortality than

N. apis, indicated that *N. ceranae* may be more pathogenic to *A. mellifera* than *N. apis* (Milbrath et al., 2015; Sinpoo et al., 2018). Recently, A third species has been described which is closely related to *N. apis*, named *N. neumanni.* This species was discovered in *A. mellifera* in Uganda and found to be much higher incidence than *N. apis* and *N. cereanae* in this region (Chemurot et al., 2017).

Nosema infection has been shown to affect the honey bee health. It degenerates gut epithelial cells of honey bee (Jack et al., 2016; Suwannapong, Maksong, Seanbualuang, & Benbow, 2010), impairs tissue integrity, modulates innate immunity pathways (Antunez et al., 2009; Li et al., 2018), and induces hormonal stress (Mayack, Natsopoulou, & McMahon, 2015), oxidative stress, and energetic stress in honey bee (Kurze et al., 2016; Martin-Hernandez et al., 2011; Mayack & Naug, 2010). *Nosema* also causes shorten lifespan in honey bees, therefore decreasing in bee population leading to reduction of honey production and crop products that rely on bees for pollination. *Nosema* infection also know to alter the levels of vitellogenin (Vg) (Goblirsch et al., 2013), a glycolipoprotein that is a precursor to the major yolk protein, vitellin, and is found in female bees. Larval honey bees infected with *N. ceranae* have increased vitellogenin titers as young adults and had fewer barbs on their stings (BenVau & Nieh, 2017).

The symptom of nosemosis is unclear, but in some cases, brown faecal marks may be seen on the comb and the front of the hive. The color of honey bee midgut, which is normally brown, will become whitish and swollen when honey bee get infection by *Nosema*. However, examination the midgut using light microscope is the only credible method of diagnosing the presence of *Nosema* spores infection (Goblirsch, 2017). The spores have a distinct size and ovoid morphology that sets them apart from other microbes and some skilled technicians may be able to distinguish between spores of *N. apis* and *N. ceranae* by eyes. However, microscopic analyses are not sensitive at detecting low levels of *Nosema* infection and difficult to differentiate of each *Nosema* species. The molecular methods, such as qPCR are required that can reliably differentiate between these different species of microsporidia, and it enables detection of the parasite even at very low levels of infection (Ansari, Al-Ghamdi, Nuru, Khan, & Alattal, 2017; Hamiduzzaman, Guzman-Novoa, & Goodwin, 2010; Soklic & Gregorc, 2016).

2.7 Biology of *Nosema*

Microsporidia are known to be a diverse group of obligate intracellular parasites that have been identified as the sources of many infectious diseases in vertebrates and invertebrates. These spore-forming parasites were described in the 19th century with the identification of *Nosema bombycis* in the European silkworm industry. There are currently more than 1,400 identified species of microsporidia classified into 197 genera (Pan et al., 2018). More than 150 of those species are members of the genus *Nosema* that infect insects, especially the orders of Lepidoptera and Hymenoptera (Chen et al., 2009; Keeling & Fast, 2002). *Nosema* is obligate intracellular spore-forming parasite that has been reclassified from protozoa to fungi (Stentiford et al., 2016). Only three species are known to infect honey bees. *N. apis* is a parasite of the European honey bee, *Apis mellifera* while *N. ceranae* is a parasite of the Asian honey bee, *A. cerana*, but they have been cross infection in *A. mellifera* and distribute worldwide (Chemurot et al., 2017; Fries et al., 1996). In 2017, a third species has been described which is closely related to *N. apis*, named *N. neumanni*. 16S rRNA gene of *N. neumanni* was found to be 97% similar to *N. apis*. This species was discovered in Uganda, and found to be much higher incidence than *N. apis* and *N. cereanae* (Chemurot et al., 2017) However, its virulent still unclear.

The taxonomy of *Nosema* was classified as follows:

Kingdom Fungi
Phylum Microsporidia
Class Dihaplophasea
Order Dissociodihaplophasida
Family Nosematidae
Genus *Nosema*
Species *N. apis*
N. ceranae
N. neumanni (Chemurot et al., 2017; Fries et al., 1996; Zander, 1909). Recenrly, Genus *Nosema* was recently reclassified into genus *Vairimorpha* based on the unrooted phylogenetic tree of two genetic markers (SSU rRNA and RPB1) (Tokarev et al., 2020). However, more several tissues still need to invalidate this classification (Bartolome et al., 2024).

The spores of three *Nosema* species have oval or rod shape and differ very slightly when observed under the light microscope (Ptaszyńska, Borsuk, Mułenko, & Demetraki-Paleolog, 2014). Fresh mature spores of *N. apis* measure approximately 6 x 3 µm with more than 30 polar filament coils whereas *N. ceranae* measure 4.4 x 2.2 µm with 20-23 polar filament coils (Chen et al., 2009; Fries et al., 1996). Spores of *N. neumanni* are smaller when compared to those of two species, which are 2.36 x 1.78 µm and have fewer polar filament coils, 10-12 polar filament coils (Chemurot et al., 2017). The observation of spores under Scanning Electron Microscopy (SEM) revealed that *N. apis* and *N. ceranae* spores differ in their surface structure. *N. ceranae* spores seem to be more sculptured with deeper ornamentation than those of *N. apis* (Ptaszyńska et al., 2014). The increase in infection with *Nosema* species was observed due to climatic changes. *N. ceranae* infection rate is more dominant in hot climates, while *N. apis* prevalence is high in cold climates (Özgör, Güzerin, & Keskin, 2015).

The mature spore of *Nosema* has a thick protective wall that consists of two layers, which are electron-dense proteinaceous outer layer or exospore and electron-transparent chitinous inner layer or endospore. These spore wall helps spore to resist the outer environment and can survive before it infects to the host (Yang et al., 2018). The internal surface of the endospore is lined with the plasma membrane which contains the sporoplasm (Han & Weiss, 2017). This sporoplasm is the infectious material of the spore, contains two typical nuclei (diplokaryotic), anchoring disk, membranous polaroplast, polar filament, endoplasmic reticulum, ribosomes, mitosome, and posterior vacuole.

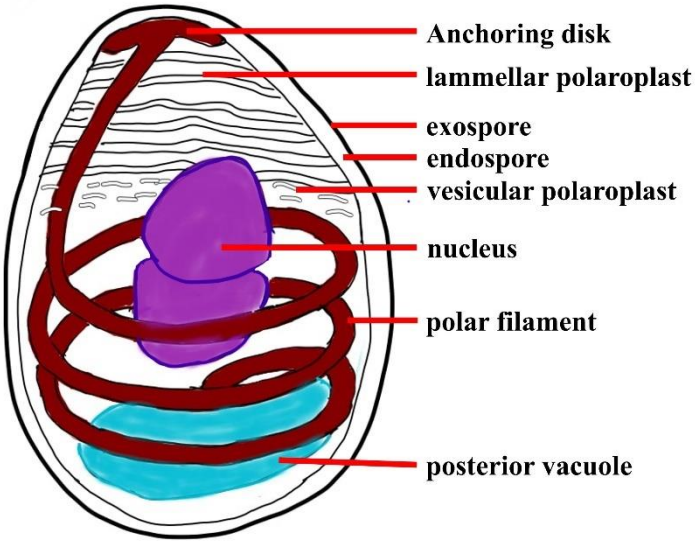

Figure 2.16 The major structures of *Nosema* spores. The figure modified from Keeling and Fast (2002).

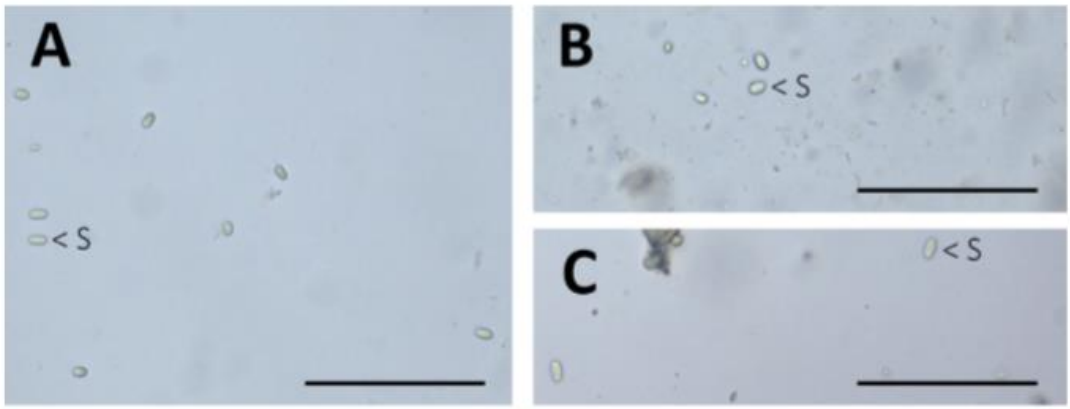

Figure 2.17 Light micrographs of spores (S) of *N. neumanni* n. sp. (A), *N. ceranae* (B) and *N. apis* (C). The scale bar is 50 µm (Chemurot et al., 2017).

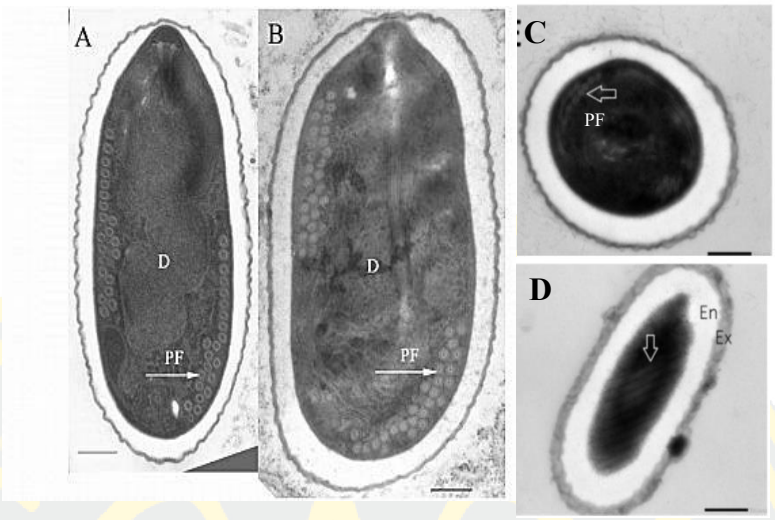

Figure 2.18 Spores of *N. ceranae* (A), *N. apis* (B), *N. neumanni* (C, D) under TEM; coil of polar filament (PF); dipolkarya (D). The scale bar is 0.5 µm (Chemurot et al., 2017; Fries, 2010).

2.8 Life cycle and transmission of *Nosema*

Nosema apis and *N. ceranae* are diplokaryotic throughout their life cycle (Goblirsch, 2017). The life cycle of these microsporidian alternates between a dispersive spore stage that is resistant to environmental stress, and intracellular replicative stages that can only take place inside the cytoplasm of an infected host cell (Heinz et al., 2014). Most microsporidia appear to have lost the ability to produce ATP, making them rely on nutrients and energy of their host (Paris, El Alaoui, Delbac, & Diogon, 2018). Hacker, Howell, Bhella, and Lucocq (2014) suggested that a range of intracellular parasites could influence the organization of host cell cytoplasm in close proximity to the infecting organism.

The general infectious cycle of microsporidia spore comprises three distinct phases. The first phase is infective phase that begins when the spore entering to honey bee midgut. They germinate and release polar tubes that transfer their sporoplasm into the cytoplasm of ventricular epithelial cells. The second phase is proliferative phase (merogony) which the sporoplasm grows and divides by binary fission or karyokinesis within the host-cell cytoplasm. They have two nuclei and are bound by a thin plasma membrane that remains in direct contact with the host cytoplasm.

Finally, meronts transform into sporonts in sporogonic phase (sporogony) (Vávra, 2014). The sporonts divide one or more times and then become sporoblasts, which having a thin spore wall and short polar filament. After that, sporont continue develop to mature spores that having thick spore wall and long polar filament (Aranedaa, Cumianb, & Moralesa, 2015; Bigliardi, Gatti, & Sacchi, 1997; Chen et al., 2009; Goblirsch, 2017; Keeling & Fast, 2002). The life cycle of *N. ceranae* was completed within 3 days after infection in honey bee, *A. mellifera*. Mature spores could be seen inside host ventricular cells with a few cells were observed to be parasitized and increased more than half of the counted cell on day 6 after infection (Higes, Garcia-Palencia, Martin-Hernandez, & Meana, 2007).

The mature spores not only accumulate in midgut epithelial cells, but also are released into the gut lumen through cell lysis (Gisder, Schuler, Horchler, Groth, & Genersch, 2017). These spores are autoinfective and can infect adjacent healthy cells within the same host (Martin-Hernandez et al., 2018). The spores can be passed to the environment through sputum and feces, providing new sources of the infection through cleaning and feeding activities in the colonies (Chen et al., 2009; Fries, Granados, & Morse, 1992). *Nosema* infection occurs mostly through ingestion of spores when they are eating contaminated food or water, and when they are cleaning up faecal material from infected bees (Forfert et al., 2015). *Nosema* spores have been found in the pollen loads of bees (Sokół & Michalczyk, 2016), which include some spewed nectar from the honey bee crop and could be spewed to other bees during food exchange, providing a mechanism for oral-oral transmission (Chen et al., 2008; Higes, Martin-Hernandez, Garrido-Bailon, Garcia-Palencia, & Meana, 2008; Smith, 2012). Moreover, *Nosema* spores can be found in the semen of male bees. Queens became infected after artificial insemination with this semen, demonstrated that *Nosema* spp. also transmit sexually in honey bees (Roberts, Evison, Baer, & Hughes, 2015).

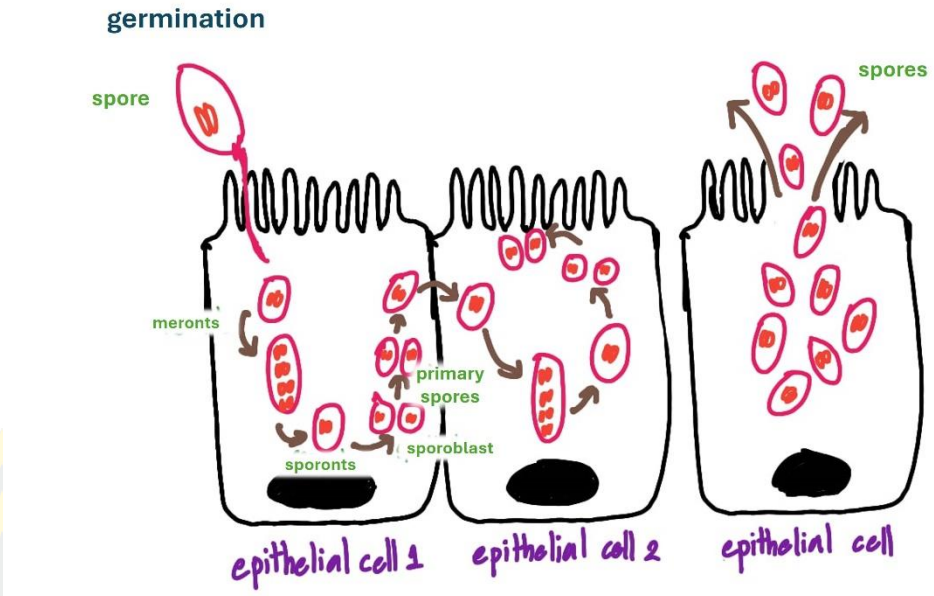

Figure 2.19 Life cycle of *Nosema* spores. The figure modified from Martin-Hernandez et al. (2018).

2.9 Wide spread of *Nosema*

Nosema apis was observed over 100 years ago in Western honey bees, *Apis mellifera* (Zander, 1909) and had become more widely distributed by cross infection from *A. mellifera* to *A. cerana*. *N. ceranae* was first isolated from the Asian honey bee, *A. cerana* in Beijing, China (Fries et al., 1996). After *N. ceranae* was observed, it was found in managed *A. mellifera* colonies worldwide (Klee et al., 2007). Infection by *N. ceranae* has been reported in a number of European countries which are Spain, France, Germany, Switzerland, Denmark, Finland, Greece, Hungary, Holland, United Kingdom, Italy, Serbia, Poland, Slovenia, Bosnia Herzegovina and Sweden (Hges, Martín-Hernández, & Meana, 2010; Higes, Martin, & Meana, 2006; Jabal-Uriel et al., 2022; Klee et al., 2007), as well as in other continents such as Saudi Arabia, Canada, Mexico, central USA, Japan, Taiwan, Vietnam and Thailand (Ansari et al., 2017; Guerrero-Molina, Correa-Benitez, Hamiduzzaman, & Guzman-Novoa, 2016; Huang, Jiang, Chen, & Wang, 2007; Klee et al., 2007; Suwannapong et al., 2010; Williams, Shafer, Rogers, Shutler, & Stewart, 2008; Yoshiyama & Kimura, 2011). In Mexico, *N. ceranae* was found to infect *A. mellifera* samples that collected in 1995-1996. This indicates that *N. ceranae* is a parasite of honey bees in Mexico since at least 1995

(Guerrero-Molina et al., 2016). In Canada and central USA, first detection of *N. ceranae* in *A. mellifera* was also reported in 2006 (Williams et al., 2008). In Saudi Arabia, the high prevalence of *N. ceranae* was present in 95 % together with the absence of *N. apis* infection (Ansari et al., 2017) same as Chen et al. (2008), who found only *N. ceranae* infection in honey bee samples collected between 1995 and 2007 from 12 states of the U.S. The geographical distribution of *N. apis* is apparently decreasing in some regions, while there are numerous reports of the presence of *N. ceranae* in *A. mellifera* worldwide. Therefore, it has been hypothesized that the distribution of *N. ceranae* may be increasing, presumably replacing *N. apis* populations.

In Thailand, *N. ceranae* was first observed in *A. cerana* in 2007 by Suwannapong et al. (2011)b, and can be found infecting *A. florea* and *A. dorsata* (Chaimanee et al., 2010; Suwannapong et al., 2010). Moreover, *Nosema* spores can be found in pollen and bee bread from pollen storage area in the comb (Peukpiboon, Benbow, & Suwannapong, 2017; Sokół & Michalczyk, 2016) and also found in honey. The presence of *Nosema* spores in pollen and bee bread must be due to self-contamination of foragers during pollen collection. Pollen may contaminate when forager bees moisten the forelegs with the protruding tongue and brush the pollen grains from the front body. The spores inside its body may become mixed with the pollen because the spores can either come directly from bee intestine after regurgitation or is present in the saliva. Therefore, Pollen and honey may be a potential source of infection (Sokół & Michalczyk, 2016; Suwannapong et al., 2011a).

2.10 Effect of *Nosema* on honey bees

Nosema can infect all honey bee castes that composed of a queen, drones and workers, but the pathological effects of this microsporidian have been mainly investigated in adult worker bees (Alaux et al., 2011; Naudi et al., 2022). However, experimental infections provided that larvae can also be infected (BenVau & Nieh, 2017; Eiri et al., 2015). After spore was ingested, the spores invade and develop within the cytoplasm of the ventricular epithelial cells for generate more spores. *Nosema* infection causes digestive disorder, swollen and broken apical parts of

epithelium cell of the midgut that negatively effect on health of both individual and colony levels *(Suwannapong* et al., 2010; Suwannapong et al., 2011b).

Nosema directly draws energy from host for their proliferation and also damage the ventricular cells of the midgut. These parasites often exert energetic stress on their host and cause higher mortality rate (Goblirsch, 2017). There was shown that honey bees infected with *Nosema* had higher hunger levels and lower trehalose levels in bee hemolymph, consequently affected to their flight ability (Martin-Hernandez et al., 2011; Mayack & Naug, 2009, 2010). *Nosema*-infected honey bees quickly altered their flight behavior and performed more flights of shorter duration (Dosselli, Grassl, Carson, Simmons, & Baer, 2016). More fight activities of *Nosema*-infected bees result from more hunger, but the flight capacity is low due to lower energy demands, which may cause foragers to fail to return to their hive (Wells et al., 2016). *Nosema* also changed the ethyl oleate (EO) levels in infected bees. This pheromone produced by workers and its function was to regulate behavioral maturation of nurse bees to foragers. Infected bees had more elevated EO titers and showed precocious than healthy bees (Dussaubat et al., 2013). Changes in vitellogenin (Vg) and juvenile hormone (JH) also contributed to precocious foraging. *Nosema* infection induced workers to forage earlier by lowering Vg while increasing JH titers (Goblirsch et al., 2013). The yolk precursor protein, Vg (Tikhonov et al*.,* 2006), and the endocrine factor, JH, are hypothesied to be physiological regulators of behavioral development in honey bee workers. Nurse bees have high Vg in fat body and low JH in hemolymph, whereas foragers have low Vg and high JH. (Goblirsch et al., 2013; Paris et al., 2018). Furthemore, *Nosema*-infected bees demonstrated improved odor learning and memory capacity at nurse age. This impact may result in precocious foraging (Gage et al., 2018). Larval honey bees infected with *N. ceranae* showed higher Vg titers as young adults. Bees infected as larvae had lower adult longevity (Eiri et al., 2015). Larvae infected with *N. ceranae* might have shown increased hunger and consumed more royal jelly, resulting in a more queen-like morphology which had fewer barbs on their stings (BenVau & Nieh, 2017).

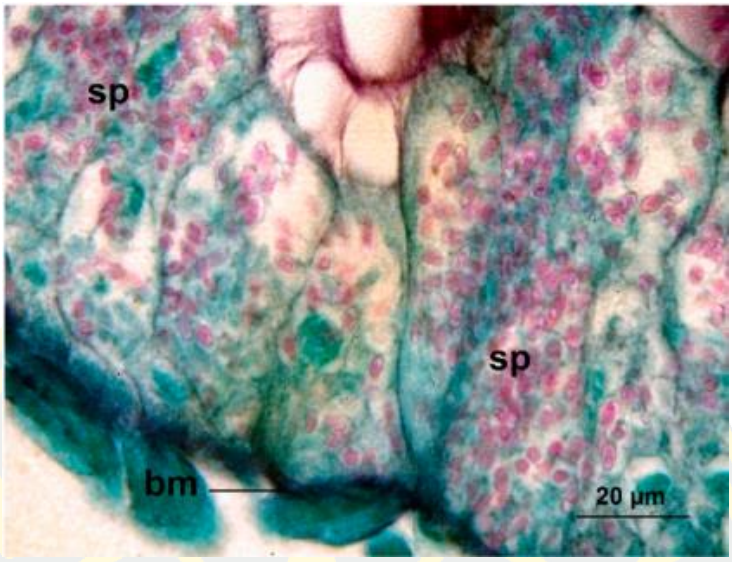

Figure 2.20 Light micrograph of cross section of midgut of *A. dorsata* heavily infected with *N. ceranae* (1,000×). Basement membrane (bm), *N. ceranae* spore (sp) (Ponkit et al., 2021).

Malone and Gatehouse (1998) reported that *N. apis* disrupted the digestive abilities of honey bees by reducing proteolytic capability. The infection caused lower levels of trypsin, chymotrypsin, elastase and leucine aminopeptidase in honey bees. Infection by *N. ceranae* also causes lower activity of total proteolytic enzyme in honey bee midgut (Ponkit et al, 2021). These reduced the ability of protein digestion that might contribute to lack of hypopharyngeal glands development. Hypopharyngeal gland protein production of honey bee was affected by *N. ceranae* (Naree et al., 2021a; Ponkit et al, 2021). These glands are a pairs of food brood glands located in the honey bee head beside the compound eyes. The glands consist of many acini secreted proteinaceous secretions which are fed to the larvae, queen and drones (Suwannapong et al., 2011a). Hypopharyngeal gland development is also influenced by age or role of worker bees. It is fully developed in nurse bees and become degenerate in older foragers and guard bees. However, the development of this glands also depends on the colony conditions such as honey bee diet and the presence of brood in the colony (Jack et al., 2016; Suwannapong et al., 2011a). *Nosema*-infected bees had lower protein production of hypopharyngeal glands that might be a result of the poor development of the glands (Der-l wang & Moeller, 1971; Naree et al., 2021c;

Suwannapong et al., 2010; Suwannapong et al., 2011b), and ability of pollen digestion (Jack et al., 2016). Genes encoding major royal jelly proteins was also down-regulated during the proliferation of *N. ceranae*. Furthermore, *N. ceranae* infection could disturb other metabolic pathways as carbohydrate, amino acid and lipid levels also decreased (Badaoui et al., 2017).

According to the honey bee's midgut is the main site of *N. ceranae* proliferation and plays important role in immunological response. Honey bee's innate immune systems vary in response to *Nosema* infection (Sinpoo et al., 2018). The expression of antimicrobial peptides: abaecin, apidaecin and hymenoptaecin in Asian honey bees (*A. cerana, A. dorsata* and *A. florea*) were higher than *A. mellifera* in both of *Nosema*-infected bees and non-infected bees, whereas abaecin was not impacted by the infection (Chaimanee et al., 2013). On the other hand, Antunez et al. (2009) reveal that the expression of abaecin was suppressed in *A. mellifera* workers after infected by *N. ceranae*. Vidau et al. (2014) reported that *Nosema*-infected bees have changed the expression of specific proteins in the midgut compared to non-infected bees. Those proteins are engaged in the key host biological functions such as energy production, innate immunity, and protein regulation, suggesting that *N. ceranae* can alter the expression of the honey bee midgut proteome to create an environment suitable for parasite development.

Nosema inhibits apoptosis, a defense mechanism against intracellular parasites, for its own advantage. The number of apoptotic cells of bee midgut gradually decreased in *Nosema*-infected bees (Kurze et al., 2018; Martin-Hernandez et al., 2017). The three apoptosis-related genes (NFAT, R1, and REL) were down-regulated in response to *Nosema* infection (Sinpoo et al., 2018), whereas *buffy* (encoding a pro-survival protein) and *BIRC5* (belonging to the inhibitor apoptosis protein family) were up-regulated (Martin-Hernandez et al., 2017). The pathogenicity of *Nosema* spp. on honey bee development and physiology can be influenced by the age at which a bee becomes infected. Roberts and Hughes (2014) found that workers infected at an older age had higher survival rates but lower ability to generate an immune response than younger. However, they are more likely to become infected at the colony level than younger workers (Jack et al., 2016).

The queen bee lays eggs and regulates the colony via pheromones produced by mandibular glands (Suwannapong et al., 2011a). Infection with *N. ceranae* can affect Vg titer, which is an indicator of fertility, longevity, antioxidant capacity, and queen mandibular pheromones (QMP). In *Nosema* infected queen, the QMP (9-ODA and 9-HAD) were higher compared to healthy queens. The increase in QMP may have an impact on the interactions between queen and workers, as queen with lower QMP is more appealing and groomed by workers than queen with higher QMP. The physiological and QMP changes also influence the queen's ability to mate and be attractive to drones (Alaux et al., 2011; Richard, Tarpy, & Grozinger, 2007). Moreover, *Nosema* can infect both immature and mature drones, with effects on fertility and longevity in older drones (Peng et al., 2015; Traver & Fell, 2011). It has been shown that *Nosema* infection incurs energetic costs in drones. They starved faster than controls and showed different patterns of flight activity in the field. They did fewer long flights and flew for shorter periods of time, suggesting that they are less likely to have an opportunity to mate than healthy drones (Holt, Villar, Cheng, Song, & Grozinger, 2018).

2.11 Energetic stress in honey bees

Honey bees need mainly carbohydrates and sugars in nectar or honeydew to provide energy for foraging and other activities. They also need protein, lipids, vitamins, and minerals that come from pollen for growth (Brodschneider & Crailsheim, 2010; Tsuruda, Basu, Sagili, 2021). Carbohydrates, such as glucose is a major energy fuel for honey bees, and trehalose is a major carbohydrate storage molecule in honey bee hemolymph (Zoltowska, Lipinski, Dmitryjuk, & Myszka, 2005). When honey bees are stressed by stressors such as pathogens, climate change, which frequently cause increased metabolic demands (Bordier et al., 2017), they must increase their mobility and utilize energy reserves to cope with the sudden increase of metabolic demand. As soon as the stress has occurred in honey bee, it will releases octopamine (OA) and dopamine (DA) within the brain, that acting as neurotransmitters. OA is subsequently released into the hemolymph from neurohemal cells to act on many organs and coordinate a physiological response to the stressor (Even, Devaud, & Barron, 2012). It has an impact on honey bee glycogenolysis,

behavior, reproduction, and stress resistance (Bordier, Klein, Le Conte, Barron, & Alaux, 2018; Wang, Kaftanoglu, Brent, Page, & Amdam, 2016). Then, the activation of the neurosecretory cells of the corpora cardiaca (CC) causes the release of several neurohormones such as adipokinetic hormones (AKH), corazonin (Crz), and possibly diuretic hormone-I (DH) into the hemolymph to mobilize energy from fat body as well as from the crop of honey bee (Even et al., 2012).

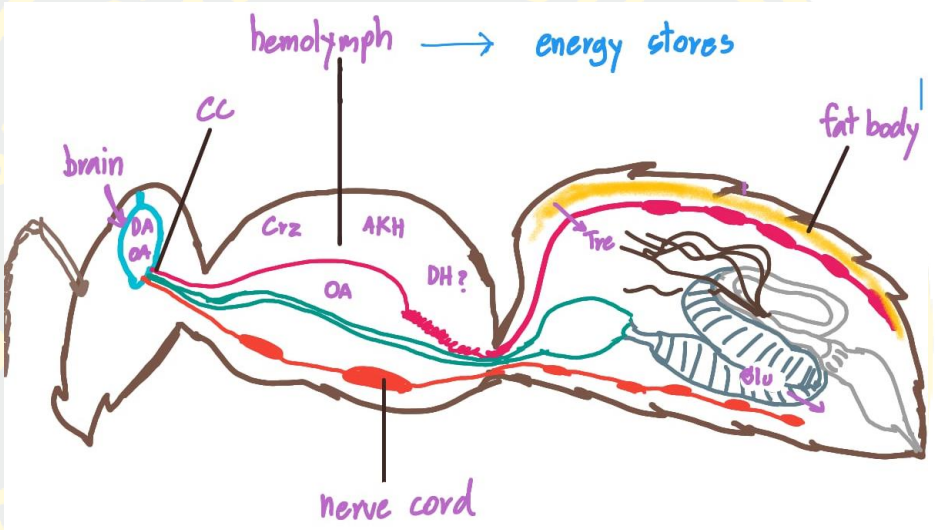

Figure 2.21 Hypothesized model of the general stress system in the honey bee; the octopamine (OA), dopamine (DA), corpora cardiaca (CC), corazonin (Crz), adipokinetic hormone (AKH), and possibly diuretic hormone-I (DH). The figure modified from Even et al. (2012).

Trehalose is stored in the fat body and released into the bee's hemolymph to be metabolized into glucose, which enters the Krebs cycle and produces ATP (Aliferis, Copley, & Jabaji, 2012). However, honey bees appear to have a specific mechanism for regulating hemolymph sugar levels. When trehalose levels drop, the proventriculus contracts, allowing nectar to flow from the crop to the midgut. In the midgut, sucrose in nectar is metabolized into fructose and glucose before being transported to the hemolymph (Even et al., 2012). Thus, the levels of fructose and glucose will increase, maintaining a stable sugar concentration.

Some pathogens have a direct influence on nutrition in honey bee. The gut parasite *Nosema* directly draws energy from host for its growth and reproduction, exerts an energetic stress on their host and interferes with digestion. These parasites lack ATP-producing mitochondria making them completely dependent on the host cell for energy (Paris et al., 2018), and their vegetative states may re-organize host cell mitochondria from which they obtain ATP. Thus, infections are energetically costly for the hosts (Holt & Grozinger, 2016). Moreover, the epithelial layer of honey bee midgut is damaged by *Nosema* proliferation. Therefore, the production of digestive enzymes and the absorption of food of honey bee is impaired (Ponkit et al., 2021; Soklic & Gregorc, 2016), causing poor nutrition in honey bee leading to honey bee has lower trehalose levels in hemolymph (Ponkit et al., 2021; Suwannapong et al., 2018) and become more hunger. Mayack and Naug (2009) showed that honey bees infected by *N. ceranae* had a higher hunger level, and reduced honey bee lifespan. They also showed *N. ceranae* could cause lower sugar level in bee hemolymph of an individual forager by measuring the trehalose. The trehalose level of infected bees declined at a faster rate while their glucose level was maintained at a quantity comparable to uninfected bees (Mayack & Naug, 2009, 2010). Increasing foraging duration and decreasing flight frequencies had been reported in infected honey bees (Dosselli et al., 2016; Wells et al., 2016). The decline of foraging efficiency is likely to be a result of imposed nutritional and energetic stress from infection. Nevertheless, there was no such association in *Nosema*-infected tolerant honey bees' lineage, suggesting that energy availability in tolerant honey bees was not compromised by the infection (Kurze et al., 2016).

Honey bee energy are not only imposed by pathogens directly but may also be a result of immune function. The activation of immune responses assumed to be costly, and honey bee could get energetic stress due to costly maintenance of an immune response (Dosselli et al., 2016). It has been reported that immune response in honey bee could compromise foraging performance due to increasing metabolic demands. Bordier et al. (2018) reported honey bees challenged by stressor such as immune challenge reduced pollen foraging and increased the duration of trips in pollen foragers. Stress also reduced the amount of OA in the brain of pollen foragers. The drop in OA level is in accordance with the behavioral changes observed in pollen

foragers after stress exposure. The author clarified that stressed bees might prefer to forage for resources that are rich in carbohydrates to overcome the energetic cost of the stress. These problem can cause a nutritional imbalance with a pollen deficit at the colony level, and thereby affect colony development (Bordier et al., 2018).

2.12 Immune defense responses of honey bees

Social life is generally associated with increased exposure to pathogens and the risk of disease transmission. Pathogens can spread rapidly in social insect colonies from close contacts among nestmates, high population density and frequent physical contact like food sharing (Baracchi, Fadda, & Turillazzi, 2012). In order to resist pathogens, social insects have evolved both individual and group strategies (social immunity) to defend against pathogens, parasites and pests (DeGrandi-Hoffman & Chen, 2015).

Social immunity describes cooperative behavioral defenses among members of a social group that effectively reduce disease and parasite transmission at the colony level (Simone-Finstrom, 2017). Honey bees also show special types of behavioral adaptations such as enrichment with antimicrobial material (Drescher, Klein, Neumann, Yanez, & Leonhardt, 2017), grooming, hygienic behavior (Nganso et al., 2017), heat balling (Gu, Meng, Tan, Dong, & Nieh, 2021; Ken et al., 2005), undertaking (Sun & Zhou, 2013), self-removing (Negri et al., 2016; Simone-Finstrom, 2017), and antiseptic enzymatic secretions (Jones, Shipley, & Arnold, 2018).

In individual level, honey bees have several mechanisms for defense against parasites and pathogens. Antimicrobial secretions on their exterior and gut environment are the first barriers of defense to prevent pathogen invasion. If a parasite or a pathogen move beyond these defenses, honey bees can protect themselves by activating their immune responses (Negri et al., 2017; Sinpoo et al., 2018). The immune system of honey bees possesses from four immune pathways: Toll (transmembrane signal transducing pathway), Imd (immune deficiency), JNK (intracellular signaling pathways) and JAK/STAT (Janus kinase/signal transducers and activators of transcription) (Chaimanee & Chantawannakul, 2016; Evans et al., 2006; Sinpoo et al., 2018; Xing et al., 2021). In insects, innate immunity is generally divided

into two main categories, which are cellular immune responses and humoral immune responses.

Cellular immunity is comprised by cell-mediated responses like phagocytosis, nodulation, melanization and encapsulation (Dubovskiy, Kryukova, Glupov, & Ratcliffe, 2016). The effector cells of the cellular immunity are the blood cells called hemocytes that transform from resting non-adherent cells into activated adherent cells when recognizing a surface as foreign (Negri et al., 2016). Phagocytosis is a rapid, cellular cleaning process of the hemolymph, where individual hemocytes engulf small pathogens like bacteria, yeast and dead cells. It is initiated when a foreign is recognized and bound by proteins in the plasma membrane of the phagocyte. The foreign is then internalized into a membrane-delimited phagosome and fuses with a lysosome that contain hydrolytic enzymes for the digestion (Hillyer, 2016). Nodulation and encapsulation involve the coordinated adherence of granulocytes that surrounds the large foreign (Negri et al., 2016). After granulocytes attach to form a layer of cells that surrounds the foreign, the granulocytes then release their contents, which often destroying the foreign, and releasing prophenoloxidase (proPO). The proPO cascade takes part in the melanin synthesis by activating the melanization of the nodules and capsules attached to the surface of the pathogens. During melanization, some reactive oxygen (ROS) and nitrogen species are generated and take part in destruction of the pathogens (Hillyer, 2016).

Humoral immunity is the most important defense system in insects including honey bees. This defense mechanism involves the synthesis of antimicrobial peptides (AMPs) that accumulate in the hemolymph against bacteria, fungi, parasites and viruses. AMPs are small peptide that generally made up of 10–50 amino acid with an overall positive charge (Chung & Khanum, 2017). AMPs can be classified in many different ways, which can be based on structure, sequence, size and mechanism of action (Kumar, Kizhakkedathu, & Straus, 2018). A classification based on structural features results in three major classes: β-sheet, α-helical and mixed or coil (Lee et al., 2015), with the first two classes being the most common in nature.

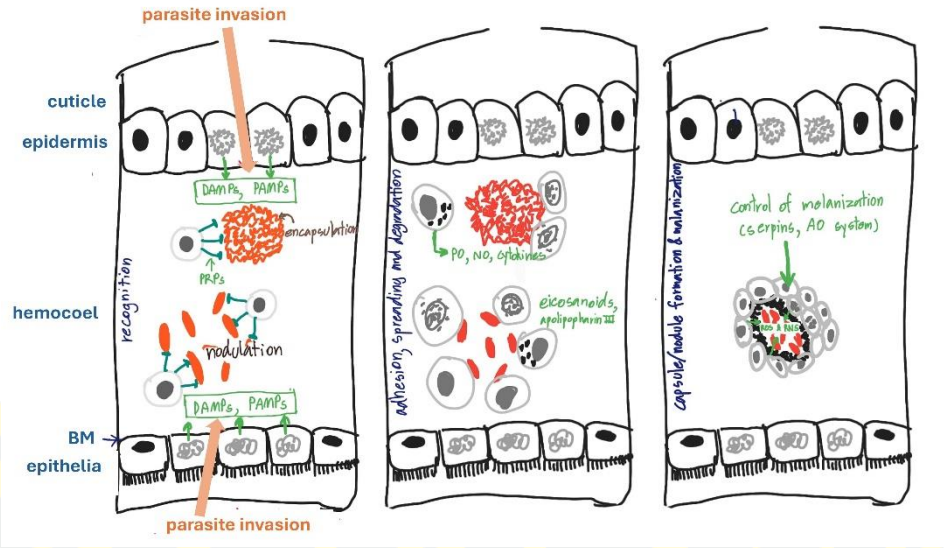

Figure 2.22 Schematic of capsule/nodule formation in insects; pattern recognition receptors (PRRs), damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), nitric oxide (NO), reactive oxygen species (ROS), reactive nitrogen species (RNS), phenoloxidase (PO), antioxidant (AO), basement membrane (BM). The figure modified from Dubovskiy et al. (2016).

AMPs exhibit rapid killing and a broad spectrum of activity against Gram-positive and Gram-negative bacteria, fungi, parasites, enveloped viruses and tumor cells (Zhang & Gallo, 2016). The positive charged peptides interact directly with the negatively charged cellular membranes of pathogens, resulting in increasing of membrane permeability, which leads to a rapid cell death. There are four different models describing possible AMPs modes of action: (1) Toroidal-pore model, initial binding of peptide to the membrane is followed by aggregation of peptides, causing the lipid moieties of outer and inner membranes to bend continuously, forming a continuous channel lined by multiple peptide units. (2) The barrel-stave model differs from the toroidal-pore model in that the peptide monomers inserted into the cell membrane are arranged parallel to the phospholipid molecules of the membrane. The lumen of the transmembrane channel will thus be lined by the hydrophilic side of the peptides, while the hydrophobic side will be interacting with the lipid core of the bilayers. This channel causing leakage of cytoplasmic material and death of the cell.

(3) The carpet model, AMPs cover the cell surface in a carpet-like manner, induce local weaknesses disintegrating the membrane structure. (4) The aggregate model, AMPs will bind to the anionic cytoplasmic membrane, causing the peptides and lipids to form a peptide-lipid complex micelle which allowing ions and intracellular contents to leak out, and then cause cell death (Le, Fang, & Sekaran, 2017; Li et al., 2018; Travkova, Moehwald, & Brezesinski, 2017; Zhang et al., 2021). Moreover, some small AMPs can kill pathogens without changing the membrane integrity. It can bind DNA, RNA and proteins, inhibiting DNA replication and protein synthesis of the pathogens (Bahar & Ren, 2013; Zhang et al., 2021).

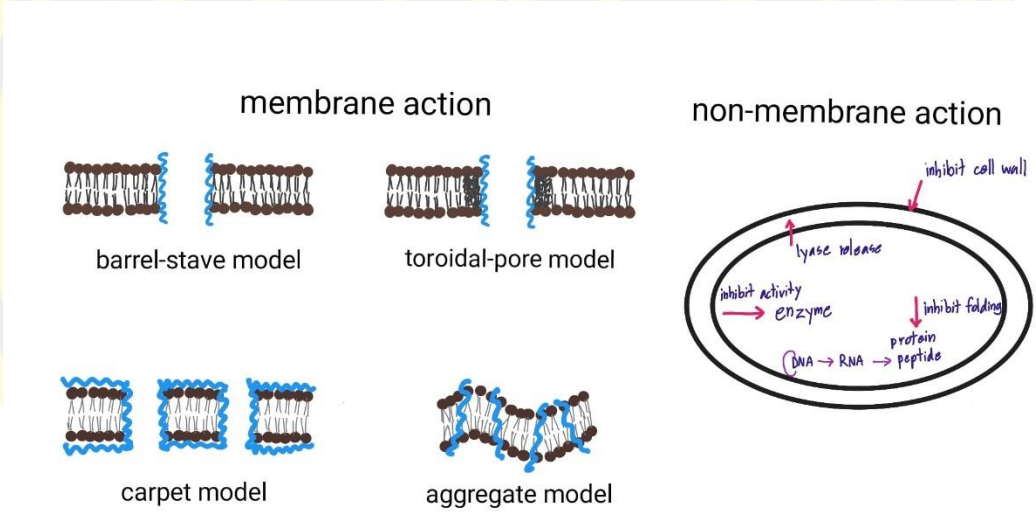

Figure 2.23 Schematic of some action mechanisms of membrane active AMPs. The figure modified form Zhang et al. (2021).

Some AMPs can be used in disease control, either to treat infected hosts, or to prevent disease transmission by interfering with parasites in their insect vectors (Li, Xiang, Zhang, Huang, & Su, 2012). In honey bee, the AMPs consist of at least four peptides which are apidaecin, abaecin, hymenoptaecin, and defensin (Pluta & Sokol, 2020; Sinpoo et al., 2018). Most AMPs are produced in the fat body or hemocytes and are secreted subsequently into the hemolymph (Rolf & Reynolds, 2009), where they diffuse to the site of infection and attack components of the pathogen cells (Mowlds, Barron, & Kavanagh, 2008).

2.13 Control of *Nosema* disease

Heating hive equipment at 49 °C for 24 hours can kill *Nosema* spores. Fumigation with acetic acid is also effective, especially when the honey bees are transferred from contaminated equipment to fumigated equipments. Fumigation with ethylene oxide (100 mg ETO/1 for 24 hours at 37.8 °C) has also been demonstrated to kill *Nosema* spores on the honey bee combs (Shimanuki, Knox, Furgala, Caron, & Williams, 1992). Gamma irradiation of 25 kGy over 9 hours 45 min can effectively inactivate *N. ceranae* spores. Honey bees fed with irradiated spores are slightly lower in spore counts and have the lowest germination (Simone-Finstrom, Aronstein, Goblirsch, Rinkevich, & de Guzman, 2018). Treatment with ozone (O_3) also reduced the viability of *Nosema* spores and other parasites in pollen, but Ozone treatment appeared to be less effective than gamma irradiation. However, both methods did not eliminate parasites completely (Graystock et al., 2016). This indicated that some irradiated spores may remain infectious after treatment (Simone-Finstrom et al., 2018). Oxalic acid (OA) is one of the strongest organic acids and it has been used for control *Nosema* in honey bees. Nanetti, Rodriguez-Garcia, Meana, Martin-Hernandez, and Higes (2015) demonstrated that 0.25 M oxalic acid solution administered to the bees in the form of sugar syrup reduced the spore load in both laboratory and field condition. They explained that the high dissociation constant and the aptitude to chelating metallic captions might change the chemical environment of the ventriculum and impair the intraluminal cycle of *Nosema*. Gisder and Genersch (2015) presented the chemicals such as metronidazole and tinidazole could inhibit *N. ceranae* proliferation, which is based on *N. ceranae* infected cultured cells. However, these compounds just inhibit active infections within bee midgut cells but will not kill spores contaminating colonies.

Fumagillin is the only antibiotic that approved for control of *Nosema* disease in honey bee and has been popularly used in United States apiculture. This chemical is obtained from the fungus *Aspergillus fumigatus* and that has been applied against microsporidian infections and diseases in apiculture and in human medicine (van den Heever, Thompson, Curtis, Ibrahim, & Pernal, 2014). Fumagillin is currently used for treatment of human microsporidiosis and certain cancers. It is known to interact with the MetAP2 enzyme, which is involved in protein maturation and post translation

processes (Lowther & Matthews, 2000; van den Heever et al., 2014). This chemical inhibits the enzyme methionine aminopeptidase-2 (MetAP2) (Guruceaga et al., 2020) and is known to block MetAP2 in a microsporidian pathogen of humans, *Encephalitozoon cuniculi*.

Spraying of 30 mg of fumagillin in 100 ml of sugar syrup 1:1 over honey bees and feeding of 90 mg of fumagillin in 250 ml of sugar syrup 1:1 using a feeder can reduce *Nosema* spore load (Mendoza et al., 2017). Although, treatment with fumagillin can inhibit *Nosema* spores growth, but it has a little effect on colony (Giacobino et al., 2016; Maistrello et al., 2008). Webster (1994) reported that honey bee queens feeding with fumagillin had been shown to have shorter lifespans. In addition to having negative effects on host physiology, fumagillin increases management costs, and residues may persist in the hive, posing risks to human health through honey consumption (van den Heever, Thompson, Curtis, & Pernal, 2015). Moreover, fumagillin persists inside hives and degrades over time. Therefore, *N. ceranae* infections can reemerge in colonies within 6 months after treatment, which is partly governed by how rapidly the fumagillin treatment is consumed by the colony and how quickly the chemical breaks down (Holt & Grozinger, 2016; Huang et al., 2013).

For resolve those problems, many natural compounds such as thymol, vetiver essential oil, lysozyme and resveratrol have been tested for the control of *Nosema*. All substances suitable for treating *Nosema* disease in honey bees without causing toxic effects on adult bees (Maistrello et al., 2008). Porrini et al. (2010) suggested the use of bacterial metabolites produced by *Bacillus* and *Enterococcus* strains which isolated from honey bee midgut and honey for control *Nosema*. A compound produced by *B. subtilis* could reduce the *Nosema* load and did not toxic for honey bee. Organic acids produced by *Lactobacillus johnsonii* CRL 1647 could reduce the intensity of the pathogen and more effective when combined with fumagillin (Maggi et al., 2013). Porrini et al. (2011) studied bioactivity of plant extracts, *Laurus nobilis*, and found that 1% concentration of extract could inhibit *N. ceranae* development in *A. mellifera*.

The essential oils of Chilean plant species, such as *Cryptocarya alba* had been shown to have antifungal activity against *N. ceranae.* The major compounds

separated from *C. alba* oil, which are α-terpineol, eucalyptol and *β*-phellandrene, also had significant effects against *N. ceranae* in *A. mellifera*. However, the antifungal effect of the complete essential oil on *N. ceranae* was the most effective than the effect of *α*-terpineol, eucalyptol or *β*- phellandrene separately. This essential oil appears to be nontoxic to *A. mellifera* adults, showing that *C. alba* oil may be a candidate for the treatment or prevention of nosemosis (Bravo et al., 2017). Furthermore, treatment with the natural phyto-pharmacological preparation "Nozevit" in sugar solution and pollen patties also reduced number of *Nosema* spores in honey bee colony (Gajger, Vaugrek, Pinter, & Petrinec, 2009). Glavinic et al. (2017) reported that "BEEWELL AminoPlus", a dietary supplement very rich in amino acids and vitamins, could inhibit *N. ceranae* spore growth and increased the expression of immune-related genes in honey bees. They suggested that immune-stimulation that reflects in the increase in resistance to diseases and reduced bee mortality.

Genetic products, RNA interference or RNAi is the one method used for controlling honey bee diseases caused by various parasites and pathogens. This method is a post-transcriptional gene silencing mechanism triggered by double stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The experimental ingestion of dsRNA corresponding to the sequences of *N. ceranae* putative virulence factor encoding polar tube protein 3 (ptp3) which is involved in host cell invasion, could effectively suppress the expression of the ptp3 gene in honey bees infected with *N. ceranae* and successfully reduced *Nosema* load in the midgut of honey bees. Ingestion of ptp3 dsRNA also led to improve innate immunity and lifespan in infected bees (Rodriguez-Garcia et al., 2018). However, RNAi have demonstrated off-target effects when longer dsRNA sequences are used. Therefore, it would be needed to ensure that dsRNA exposure does not negatively affect molecular processes in all honey bee castes and life stages (Holt & Grozinger, 2016).

2.14 Bee propolis

Propolis is a natural resinous substances that honey bees, stingless bee and some ant species collected from parts of plants, buds, and exudates, and are subsequently mixed with beeswax and salivary gland secretions (Sanches, Pereira, & Serrão, 2017). Honey bees use propolis for fill cracks and crevices in their hive, coat

the interior surface of the nest cavity, and block hive entrances to prevent the entry of external invaders or against weathering threats like wind and rain. Honey bees also use propolis as an “embalming” substance to cover the carcass of a hive invader, which the bees have killed but cannot transport out of the hive (Borba, Klyczek, Mogen, & Spivak, 2015; Drescher et al., 2017). Moreover, honey bee species which make an exposed nest may use propolis occasionally to strengthen the site of comb attachment on a branch and also make a ring of propolis on the branches leading to their nest to prevent ants from invading their nests (Simone-Finstrom & Spivak, 2010).

Propolis is highly complex mixture of various plant resins and wax (Drescher et al., 2017). It contains approximately 55% resinous compounds and balsam, 30% beeswax, 10% ethereal and aromatic oils, 5% bee pollen and other chemical compounds. The chemicals compounds of propolis are composed of a variety of water insoluble compounds, such as various phenolic constitutions and terpenoids. It also contains a small fraction of volatile substances which contributes to typical propolis characteristics such as its distinctive aroma and its biological activity. However, its composition varies according to the collecting location, time, food plants, extraction process and bee species. Propolis has been reported to have several biological efficacies including anticancer, antioxidant, and anti-inflammatory activities (Pasupuleti, Sammugam, Ramesh, & Gan, 2017)

The biological activity and pharmacological properties of propolis have been investigated with a wide range of antimicrobial, antifungal, antiviral, and immunomodulatory properties (Drescher et al., 2017). The antibacterial activity of Africanized honey bee green propolis was studied by (Farnesi, Aquino-Ferreira, De Jong, Bastos, & Soares, 2009). They reported green propolis had efficient against *Micrococcus luteus, Staphylococcus aureus* and *Escherichia coli*. Borba et al. (2015) showed that the presence of a naturally constructed propolis within *A. mellifera* hive was found to reduce the expression of immune-related genes (hymenoptaecin and abaecin) without affecting levels of pathogens and parasites. They suggested that the propolis might have evolved to regulate the costs of the immune system activity by reducing the investment in immune expression when the colony is not pathogen challenged. Therefore, a decrease in energetic costs will help bees to allocate their

energy (Borba et al., 2015). Borba and Spivak (2017) demonstrated that the presence of propolis envelope in honey bee hive also helped protect the brood from bacterial pathogen infection such as *Paenibacillus larvae*, the causative agent of American foulbrood. They also found the larval food collected from colonies with a propolis envelope, had a significantly higher inhibitory activity against *P. larvae* compared to larval food from colonies without a propolis envelope. In addition, Drescher et al. (2017) reported propolis decreased deformed wing virus (DWV) titers in relation to *Varroa destructor* infestation, and it can impact on the reproducing of this parasite (Pusceddu et al., 2021). Propolis also had decreased infection intensities of *Ascophaera apis*, the causative agent of the larval disease chalkbrood (CB). And colonies increased resin foraging rates after a challenge with this fungal parasite (Simone-Finstrom & Spivak, 2012).

Stingless bees are highly eusocial bees belonging to the order Hymenoptera and one of four tribes (Meliponini) in the family Apidae. There are over 500 described species in 32 genera that can be found throughout tropical or subtropical regions around the world (Chuttong, Chanbang, Sringarm, & Burgett, 2016; Ramirez et al., 2010). In Thailand, 31 species in 10 genera have been described. They are collectively known in the vernacular as 'channarong', which is derived from 'the factory that makes cerumen or propolis' (Chuttong et al., 2016). Propolis of stingless bees differs from propolis of honey bee by adding soil or clay particles to the final mixture of plant exudates and beeswax, forming the so-called geopropolis (Alday et al., 2016).

Propolis from stingless bee has important therapeutic activities, which suggest its potential application in the pharmaceutical industry, as well as in health foods, beverages, and nutritional supplements (Campos, 2015), and found to have antimicrobial and anticancer activities (Kothal & Jayanthl, 2015). The ethanolic extract of propolis produced by Brazilian stingless bee, *Melipona quadrifasciata anthidiodes* (Mandaçaia) had higher antioxidant activity compared to green propolis produced by *A. mellifera* (Pazin et al., 2017) and it also showed good inhibitory activity against *Aspergillus niger, Candida albicans* and *Trichophyton rubrum* (Kothal & Jayanthl, 2015). Arismendi, Vargas, López, Barría, and Zapata (2018) demonstrated that methanolic extracts of stingless bee propolis (8% from Biobío regions) could decrease *N. ceranae* load in *A. mellifera* and increased the survival of infected bees.

Propolis from *Tetrigona apicalis* were also high efficiency when treated in both *N. ceranae*-infected *A. cerana* and *A. florea* workers. Propolis treated bees had significantly lower infection loads, and higher survival in comparison to untreated bees. The ethanolic propolis extract of stingless bee might have toxic effects on *N. ceranae* spores, cause abnormal structure of the spores resulting in interfering or inhibiting spore growth and development (Suwannapong et al., 2018; Yemor et al., 2016).

2.15 Chito-oligosaccharide

Chito-oligosaccharides (COS) are homo- or heterooligomers of *N*-acetylglucosamine and *D*-glucosamine that can be produced from chitin or chitosan, using chemical or enzymatic conversions. It has received considerable attention for its application in the biomedical, food, textile and chemical industries. Chitosan has high molecular weight (MW) which causes high viscosity and low solubility that limited its application. Therefore recent studies on chitosan have focused attention towards conversion to COS. COS is water-soluble due to their shorter chain lengths (generally, the MW of COS is <10 kDa) and free amino groups in D-glucosamine units which confer them positive charge, that allow to bind strongly to negatively charged surfaces (Kong, Chen, Xing, & Park, 2010). Additionally, COS have been reported to possess versatile functional properties that make them a very promising class of compounds such as antitumor activity (Azuma, Osaki, Minami, & Okamoto, 2015), immuno-enhancing (Saltykova et al., 2016), enhancement of protection against infection by some pathogens, as well as antifungal and antibacterial activities (Goy, de Britto, & Assis, 2009).

COS can be considered as potential anticancer agents which expresses tumor suppression activity against various cell lines. It can inhibit the proliferation of human lung cancer cell line Hep-G2 and promotes cells apoptosis, causing the changing of the proliferation process and efficiently improves the sensitivity of HepG2 to radioactive rays. It also reduces the percentage of S-phase and decreases DNA synthesis rate in HepG2 cells (Han, Cui, You, Xing, & Sun, 2015; Shen, Chen, Chan, Jeng, & Wang, 2009). Moreover, COS also show efficient as the inhibitor of colon

carcinoma (HCT-116) and breast carcinoma (MCF7) (El-Sayed, EL-Sayed, Wafaa, Abeer, & Nagwa, 2012).

Several mechanisms have been proposed for the antimicrobial activity by chitosan and its derivatives such as 1) chitosan and its derivatives interacts with the membrane of the fungi or bacteria. The plasma membrane damage occurs by the interaction of the positive charges of the protonated amino groups of chitosan and the negatively charged molecules of the cell membrane, which change the permeability of the cell membrane, leading to leakage of intracellular material and cell death (Mansilla et al., 2013; Verlee, Mincke, & Stevens, 2017; Wang, Zuo, Wang, Na, & Gao, 2015), 2) chitosan can inhibit microbial DNA/RNA synthesis, which leads to the disruption of protein synthesis (Marquez et al., 2013; Verlee et al., 2017), 3) chitosan also can be a chelating agent to bind nutrients and essential metals to inhibit the microbial growth (Chien, Yen, & Mau, 2016; Del Carpio-Perochena et al., 2015), 4) chitosan on the surface of the cell acts as an oxygen barrier which can inhibit the growth of aerobic bacteria (Hosseinnejad & Jafari, 2016; Yuan, Lv, Tang, Zhang, & Sun, 2016).

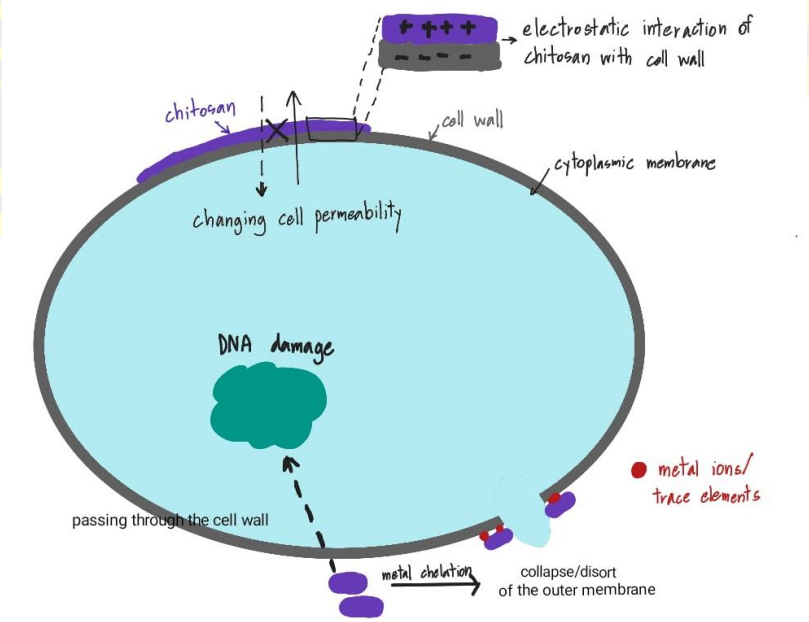

Figure 2.24 Antimicrobial mechanisms of chitosan and its derivatives. The figure modified from Hosseinnejad & Jafari (2016).

However, variations in antimicrobial efficacy of chitosan and its derivatives depends on its molecular weight (Mw), concentrations, deacetylation degree (DD), preparation method, pH and the target organism. Some studies reported increasing chitosan Mw lead to decreasing chitosan activity against *E. coli*, while in another studies showed high Mw chitosan had greater activity than low Mw chitosan (Tikhonov et al., 2006). Chitooligosaccharides (<3 kDa) has higher activity against *B. cereus* than higher Mw chitosans (628 and 100 kDa) (Fernandes, Eaton, Gomes, Pintado, & Malcata, 2009). Wang et al. (2007) demonstrated that antimicrobial activities of COS increased with increasing of deacetylation degrees, but decreased with increasing of polymerization degrees. Jeon, Park, and Kim (2001) studied the antibacterial effect of three kinds of COS, higher Mw (10 kDa), medium Mw (5 kDa), and lower Mw (1 kDa), against various bacteria including four Gram-negative bacteria (*E. coli* KCTC 1682, *E. coli* O-157 ATCC 11775, *Salmonella typhi* KCTC 2424, *Pseudomonas aeruginosa* KCTC 1750), five Gram-positive bacteria (*Streptococcus mutans* KCTC 3065, *Micrococcus luteus* KCTC 10240, *S. aureus* ATCC 6538P, *S. epidermidis* KCTC 1917, *B. subtilis* KCTC 1028), and four lactic acid bacteria (*L. bulgaricus* KCTC 3188, *L. casei* KCTC 3189, *L. fermentum* KCTC 3112, *Streptococcus faecalis* ATCC 10541). The results showed that COS with molecular weight 10 kDa has the most inhibitory effect and has more effective activity against pathogens than non-pathogens, except in the case of lactic acid bacteria. In contrast Selenius, Korpela, Salminen, and Gallego (2018) reported that COS with a degree of deacetylation (DD)≥ 90% and Mw≤ 1.5 kDa enhanced the growth of lactic acid bacteria, *L. rhamnosus* GG. Moreover, COS also could inhibit the germination of the pathogenic fungus *Botrytic cinerea* and *Mucor piriformis* (Rahman, Hjeljord, Aam, Sorlie, & Tronsmo, 2014).

Chitosan is polycationic at pH< 6 and interacts readily with negatively charged substances (Hosseinnejad & Jafari, 2016). Li, Wu, and Zhao (2016) showed that the optimal pH value for the highest bactericidal activity against *E. coli* and *S. aureus* was 6.0. Six chitosans with molecular weights of 300, 156, 72.1, 29.2, 7.1, and 3.3 kDa were studied for their antimicrobial activity against *E. coli* and *S. aureus*. At pH 5.0 and 6.0, the chitosan activity increased as the Mw increased, whereas at pH

7.0, the chitosan with Mw> 29.2 kDa greatly lost their activity (Chang, Lin, Wu, & Tsai, 2015). Those results showed that the molecular weight (Mw), concentrations, deacetylation degree, preparation method, pH and the target organism can affect the efficacy of chitosan and its derivative.

Xu et al. (2014) investigated the effects of dietary chitosan (molecular weight about 40–60 kDa) on growth performance, nutrient digestibility, and digestive enzyme activities in weaned pigs. The dietary chitosan improved digestibility of major nutrients (dry matter, crude protein, Ca, and P) and increased amylase activity of proximal jejunum but decreased lipase activity of distal jejunum. Moreover, chitosan also enhances the intestinal structure. Walsh, Sweeney, Bahar, Flynn, and O'Doherty (2012) showed that pigs fed with 10 to 50 kDa COS had a higher villous height in the duodenum and jejunum, while pigs fed with 5 to 10 kDa COS had a lower *lactobacilli* and *E. coli* population in the colon. Yousef, Pichyangkura, Soodvilai, Chatsudthipong, and Muanprasat (2012) reported that COS can prevent inflammation associated intestinal damage and mortality in mice. It has been reported that chitosan (200 kDa, 75% deacetylated) is shown to have a preadaptive effect and increase the lifespan of honey bees due to the induction of protective antioxidant and immune mechanisms (Saltykova et al., 2016), suggesting that COS might protect the ventricular epithelial cells of honey bee midgut from *Nosema* invasion. Feeding honey bees with chitosan (200kD, 75% deacetylation) can reduce *N. apis* infection (Saltykova et al., 2018). Moreover, chitosan can reduce *N. ceranae* infection when fed to worker bees in sucrose syrup (Valizadeh et al., 2021).

CHAPTER 3
MATERIALS AND METHODS

3.1 Instruments

- analytical balance (Sartorius Group, Göttingen, Germany)
- centrifuge (WiseSpin CF-10, Daihan Scientific, Gangwon-do, Korea)
- freezer (Sharp SJ-X43T, Sharp Thai Co., Ltd., Bangkok, Thailand)
- hemocytometer (BOECO, Boeckel + Co, Hamburg, Germany)
- hotplate stirrer (Ika C-MAG HS 7, Bangkok, Thailand)
- hygrometer (Barigo-8861, Schwenningen, Germany)
- incubator (Memmert IPP 260, Schwenningen, Germany)
- light microscope (Olympus CX31, Shinjuku, Tokyo, Japan)
- pH meter (Mettler Toledo F20, Mettler-Toledo GmbH, Switzerland)
- rotary microtome (Leica 2045, Wetzlar, Germany)
- slide warmer (Lab-line 26020, Illinois, USA)
- spectrophotometer (UV-1601, Shimadzu, Australia)
- stereo microscope (Olympus SZ 30, Shinjuku, Tokyo, Japan)
- vortex mixer (ONiLAB MX-S, Walnut, CA, USA)
- water bath (Memmert W 270, Gemini BV, Schwabach, Germany)

3.2 Inventory supplies

- amber glass bottle
- beakers (Pyrex, Germany)
- cuvettes (VWR international, USA)
- cylinder glass (Glassco, UK)
- filter paper (Whatman No. 1 and 4, England)
- forcep No. 3 INOX (Dumont, Switzerland)
- glass bottle (Duran, Germany)
- microcapillary (BIOMED GROUP, Thailand)
- micropipette (DLAB Scientific, China)
- microcentrifuge tubes 0.5, 1.5, and 2 ml (Eppendorf, Hamburg, Germany)

- mortar and pestle (Glassco, Haryana, India)
- parafilm M (EMS, USA)
- pipette tips (Biologix Group, USA)
- plastic pestle
- slides and cover glasses (Sail brand, China)
- staining jars
- sterile needle
- test tube (Pyrex, Germany)
- volumetric flask (Glassco, UK)

3.3 Chemicals

3.3.1 Chemicals for tissue preparation

- glacial acetic acid (Analar, England)
- picric acid (Labchem, Australia)
- formaldehyde solution (Univar, Australis)

3.3.2 Chemicals for tissue staining

- ethyl alcohol (J.T Bader, Malaysia)
- basic fuchsin (Labchem, Australia)
- distilled water
- hydrochloric acid (J.K Baker, USA)
- light green crystal (Fluka, Switzerland)
- N-butyl (RCI Labscan, Thailand)
- permount (Fisher Chemical, USA)
- phosphotungstic acid (Loba Chemie, India)
- sodium bisulfite (Sigma Andrich, USA)
- xylene (Sigma Andrich, USA)

3.3.3 Chemicals for trehalose measurement

- anthrone (Himedia, India)
- sulfuric acid
- 0.85% sodium chloride

3.3.4 Chemicals for protein measurement

- bovine serum albumin (BSA) (Sigma Andrich, USA)
- Coomassie blue 250 G (Sigma Andrich, USA)
- methanol (Sigma Andrich, USA)
- phosphoric acid (Univar, Australis)
- 0.5 M sodium hydroxide (Sigma Andrich, USA)

3.3.5 Chemicals for proteolytic enzyme activity measurement

- Tris base (Sigma Andrich, USA)
- azocasein (Sigma Andrich, USA)
- 10% trichloroacetic acid (Sigma Andrich, USA)

3.4 Specimens

3.4.1 Honey bees

- *Apis cerana* workers
- *A. dorsata* workers
- *A. florea* workers
- *A. mellifera* workers

Apis cerana, *A. dorsata*, and *A. florea* workers were collected from the colonies located in Samut Songkhram Province, Thailand while *A. mellifera* workers were collected from colonies at the honey bee research unit of Burapha University (beefamBUU), Chon Buri Province, Thailand for *N. ceranae* isolation. The frames of sealed worker broods of *A. dorsata* were obtained from wild at Samut Songkhram Province, Thailand. Moreover, frames of sealed worker broods of *A. mellifera* were obtained from three healthy colonies in an apiary located at beefamBuu. These brood frames of both *Apis* species were then kept in the incubator maintained at 34 ± 2 °C to obtain newly emerged bees for cage experiments.

3.4.2 *Nosema ceranae* spores

Nosema ceranae spores were isolated from *A. cerana*, *A. dorsata*, and *A. florea* workers collected from heavily infected colonies in Samut Songkhram

Province, Thailand. Moreover, *N. ceranae* spores were isolated from *A. mellfiera* workers collected from infected colonies in beefamBUU, Chon Buri Province, Thailand. *N. ceranae* spores obtained from each honey bee species were then propagated in *A. mellifera* workers.

3.4.3 Stingless bee propolis

Propolis was collected from three hives of stingless bee, *Tetrigona apicalis* located at agricultural extension and development center, Chiang Mai Province, Thailand in April 2021. Propolis was collected from the wall of the hive using the stainless-steel spatula and kept in a dark container to protect from the light.

3.5 Study site

The experiments were performed at the Department of Biology, Faculty of Science, Burapha University, Chon Buri, Thailand, room number BS3108, biological science building, and Department of Biology, Martin-Luther University Halle-Wittenberg, Halle (Saale), Germany.

3.6 Materials and methods

3.6.1 Experiment I: To examine the virulence of *Nosema ceranae* isolated from four honey bee species of Thailand; *Apis cerana*, *A. dorsata*, *A. florea*, and *A.mellifera* in the giant honey bee, *A. dorsata* workers.

3.6.1.1 *Nosema ceranae* spore preparation

1. *Nosema ceranae* spores were extracted separately from the 50 workers of *A. cerana*, *A. dorsata*, *A. florea,* and *A. mellifera.* Those honey bee workers were collected from *N. ceranae*-infected colonies located in honey bee research unit of Burapha University (BeefamBUU), Chon Buri Province, Thailand.

2. Ten midguts of each honey bee species were removed and transferred to a 1.5 ml microcentrifuge tube containing 200 µl of distilled water.

3. Their midguts were crushed and centrifuged at 6,000 g for 10 min (Suwannapong et al., 2018). The white sediment (*N. ceranae* spores) at the bottom

was collected and re-suspended in distilled water. This process was repeated for three times.

4. Then, the number of spores was counted under the light microscope using a hemocytometer following the method of (Cantwell, 1970).

5. Spores were then centrifuged and re-suspended in 50% sucrose solution (w/v) to obtain a concentration of 1×10^2, 1×10^3, 1×10^4, 1×10^5, 5×10^5, and 1×10^6 spores per 2 µl and kept at room temperature for infection.

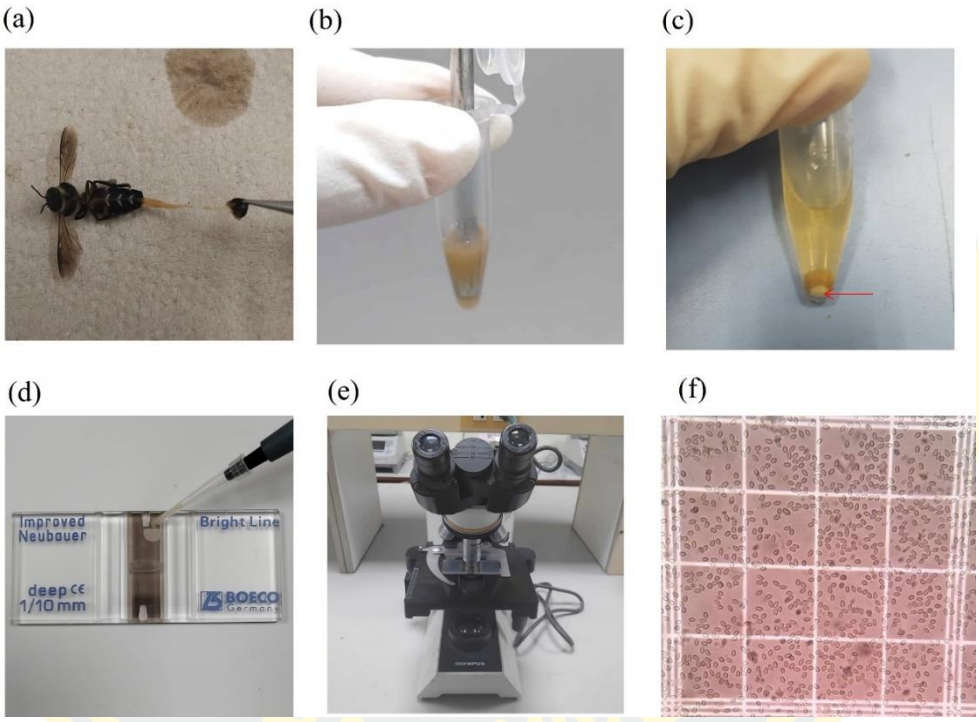

Figure 3.1 *Nosema ceranae* extraction process; honey bee's midgut removing (a), midgut homogenization (b), the white sediment (*N. ceranae* spores) at the bottom of the tube after centrifugation (c), a hemocytometer (d), a light microscope used for spores counting (e), and *N. ceranae* on the hemocytometer under a light microscope (f).

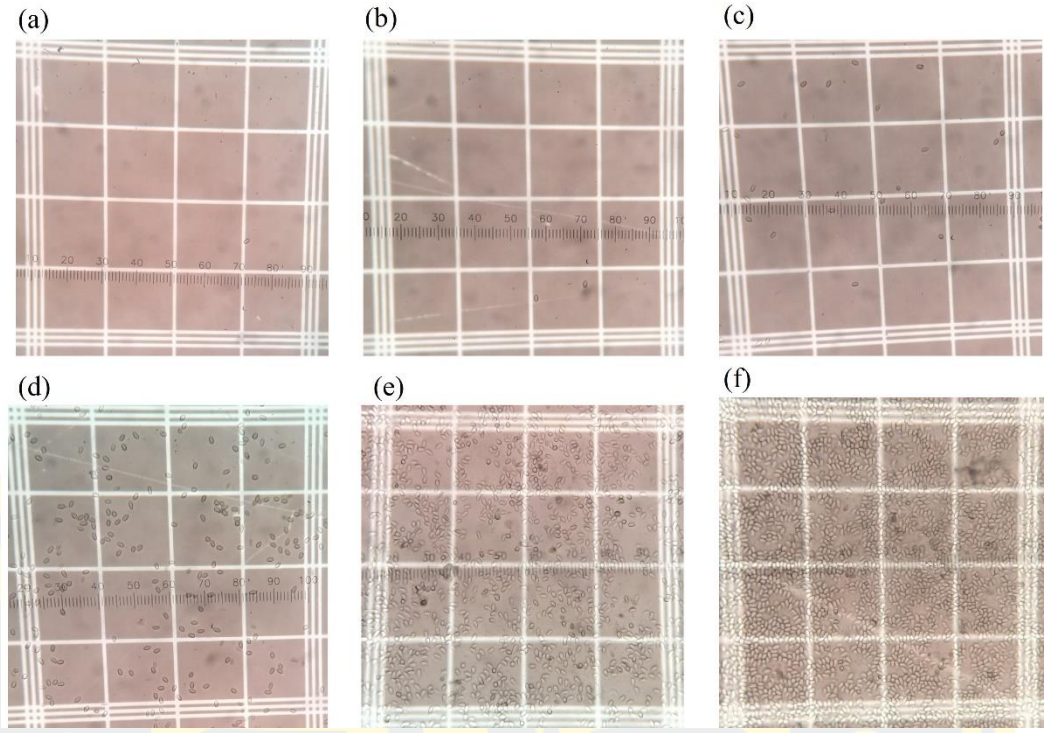

Figure 3.2 *Nosema ceranae* spores on the hemocytometer under a light microscope at different dosages; 1×10^2 spores per 2 µl (a), 1×10^3 spores per 2 µl (b), 1×10^4 spores per 2 µl (c), 1×10^5 spores per 2 µl (d), 5×10^5 spores per 2 µl (e), and 1×10^6 spores per 2 µl (f).

3.6.1.2 Newly emerged bee preparation

1. Sealed brood frames of *A. dorsata* workers were obtained from healthy *Nosema*-free colonies located in Samut Songkhram Province, Thailand.

2. The comb frames were then maintained at 34 ± 2 ˚C in the incubator (memmert IPP 260, Germany) with relative humidity (RH) of 50 - 55% to obtain newly emerged bees (≤ 24 h. old) (Figure 3.3a-c).

3.6.1.3 Experimental infection

To study the virulence, and the 50% and 100% infectious dose (ID_{50} and ID_{100}) of *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A.mellifera* in the giant honey bee, *A. dorsata* workers.

1. The newly emerged bees, *A. dorsata* workers were carefully removed and confined into bee cages (6 × 13 × 18 cm3) in groups of 50. These caged bees

were divided into 25 treatment groups with three replicated cages per group, one for control group and 24 for *A. dorsata* workers infected with *N. ceranae* isolated from four bees species at the dosage of 1×10^2, 1×10^3, 1×10^4, 1×10^5, 5×10^5, and 1×10^6 spores per bee.

2. For *N. ceranae*-infected groups, bees were force-fed with 2 µl of 50% sucrose solution (w/v) containing *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* at the dosages of 1×10^2, 1×10^3, 1×10^4, 1×10^5, 5×10^5, and 1×10^6 spores per bee.

3. For control groups, bees were force-fed with 2 µl of 50% sucrose solution (w/v) without *N. ceranae* spores.

4. Each cage was provided 2 gravity feeders, one containing water, and the other containing 50% sucrose solution (w/v) that was replenished during the experiment. The pollen mixed (60 g of pollen mixed with 17 ml of 50% sucrose solution (w/v)) was also provided to each cage.

6. A queen pheromones (QMP) strip was provided to each cage for creating environmental conditions as similar as inside the hive.

7. All experimental cages were then placed in an incubator and maintained at 34 ± 2 ˚C with 50 - 55% RH (Figure 3.3d-g).

3.6.1.4 Survival analysis

1. Dead bees from each cage were counted daily.

2. Kaplan-Meier survival curves of all treatment groups were generated by plotting the number of surviving bees against days from the initiation of the experiment.

3. The data were analyzed using a Log-rank test.

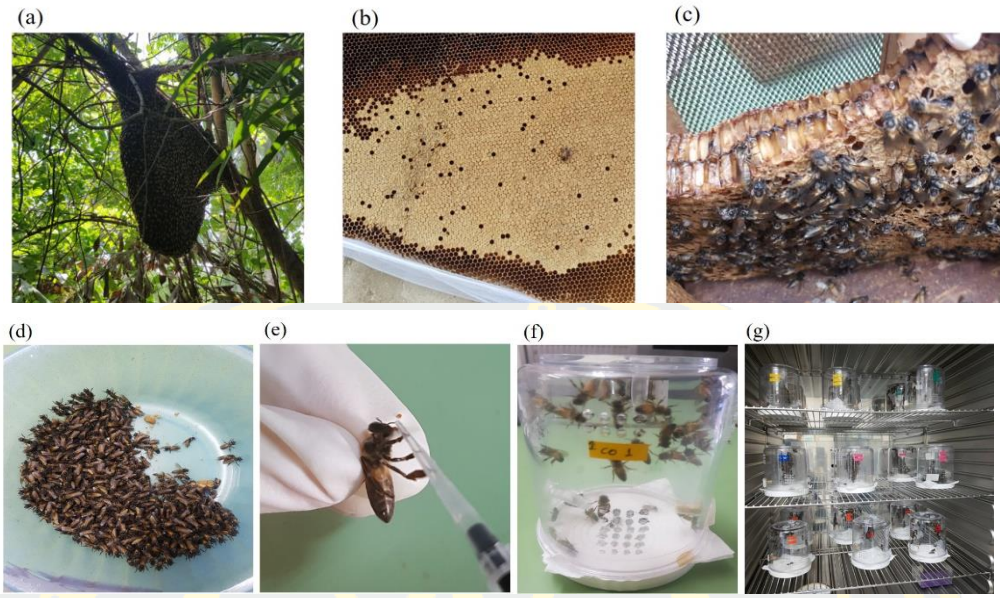

Figure 3.3 Newly emerged bees preparation and experimental infection; an *A. dorsata* nest in Samut Songkhram Province, Thailand (a), a comb with capped broods (b), newly emerged bees, *A. dorsata* (≤ 24 h-old) (c, d), force-feeding (e), and fifty bees in the experimental cage (f) maintained at 34 ± 2 °C with relative humidity between 50 - 55% (g).

3.6.1.5 Infection rate & infectivity

1. Any dead bees and alive bees were collected from each cage on days 14 post-infection (p.i.) for detecting and quantifying the spore loads per honey bee.

2. The midguts were removed individually and crushed in 100 µl of distilled water (Figure 3.4).

3. The number of *N. ceranae* of individual bees were counted under the light microscope (Olympus CH31, Japan) using a hemocytometer (BOECO, Germany). The infection rate of each treatment group was also calculated.

4. The data were represented as means ± SE. The results were analyzed using Kruskal-Wallis test, and Mann-Whitney U test were used to test the differences of median among treatment groups.

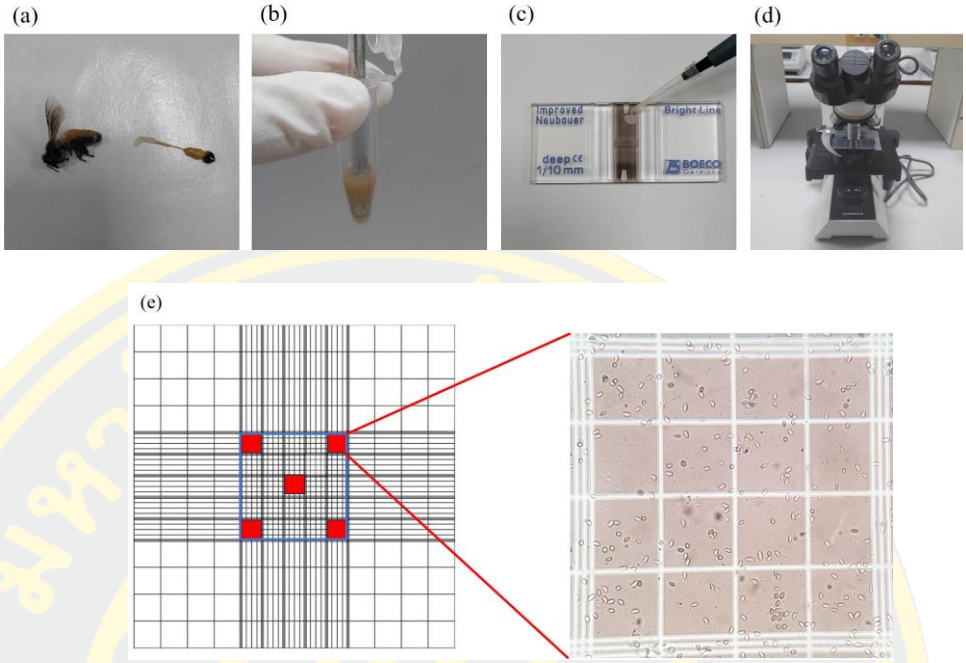

Figure 3.4 The *Nosema* spore counting process; bee midgut removing (a), midgut homogenization (b), a hemocytometer (c) and a light microscope (d) used for spore counting, and the 25 squares of hemocytometer with *N. ceranae* spores under a light microscope (e).

3.6.1.6 Food consumption

1. Food consumption was recorded every two days by weighing the feeders that containing sucrose solution, water, and pollen before and after use.

2. The mean consumption per bee was then calculated.

3.6.2 Experiment II: To investigate the effect of *N. ceranae* isolated from *A. mellifera* on energetic stress of *A. dorsata* workers using a dosage of 5×10^5 spores per bee (a dosage that results in 100% infection (ID_{100})).

3.6.2.1 *Nosema ceranae* spore preparation

1. *Nosema ceranae* spores isolated from *A. mellifera* were obtain from the experiment I.

2. The spores were then propagated in *A. mellifera* workers collected from *Nosema*-free colonies located at BeefamBUU, Chon Buri, Thailand (Figure 3.5).

3. After 14 days, ten midguts of propagated bees were removed and transferred to a 1.5 ml microcentrifuge tube containing 200 µl of distilled water.

4. Their midguts were crushed and centrifuged at 6,000 g for 10 min (Suwannapong et al., 2018). The white sediment (*N. ceranae* spores) at the bottom was collected and re-suspended in distilled water. This step was repeated for three times.

5. Then, the number of spores was counted under the light microscope using a hemocytometer following the method of (Cantwell, 1970).

6. Spores were then centrifuged and re-suspended in 50% sucrose solution (w/v) to obtain a concentration of 5×10^5 spores per 2 µl and kept at room temperature for infection.

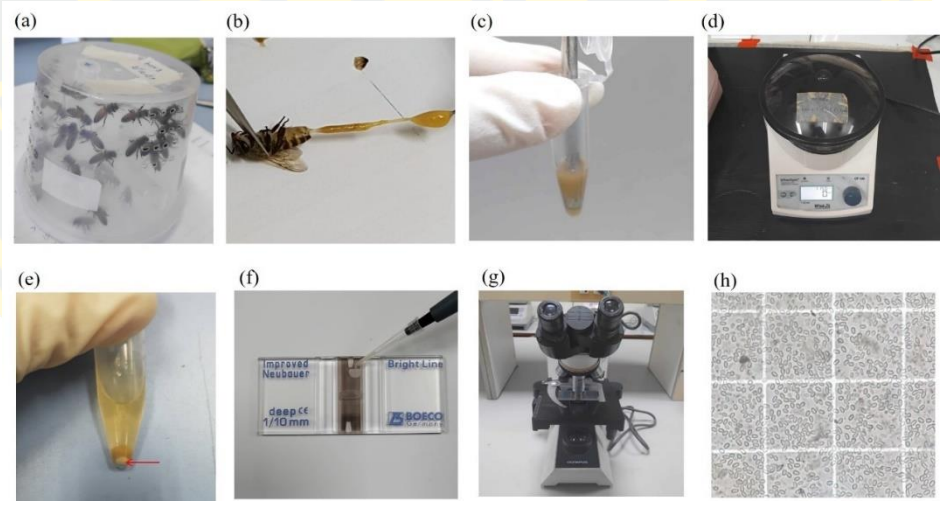

Figure 3.5 *Nosema ceranae* extraction; a cage of *N. ceranae* propagated bees, *A. mellifera* (a), midgut removing (b), midgut homogenization (c), centrifugation (d), the white sediment (*N. ceranae* spores) at the bottom of the tube after centrifugation (e), a hemocytometer (f), a light microscope (g) used for spores counting, and *N. ceranae* on the hemocytometer under a light microscope (h).

3.6.2.2 Newly emerged bee preparation

1. Sealed brood frames of *A. dorsata* worker were obtained from healthy *Nosema*-free colonies located in Samut Songkhram Province, Thailand.

2. The combs were then maintained at 34 ± 2 ˚C and 50 - 55% relative humidity (RH) to obtain newly emerged bees (≤ 24 h. old).

3.6.2.3 Experimental infection

1. Newly emerged bees were carefully removed and confined into cages (size: h = 13 cm, r = 5.25 cm) in groups of 50 with three replicated cages per treatment.

2. The experiment was divided into 2 treatment groups; one for control group and another for *N. ceranae*-infected group, with three replicate cages per group.

3. For control group, bees individually force-fed with 2 µl of 50% sucrose solution (w/v) without *N. ceranae* spores, defined as CO

4. *Nosema ceranae*-infected bee group, bees individually force-fed with 2 µl of 5×10^5 *N. ceranae* spores suspended in 50% sucrose solution, defined as NC

5. Each cage was provided 2 gravity feeders, one containing water, and the other containing 50% sucrose solution (w/v) that was replenished during the experiment. The pollen mixed (60 g of pollen mixed with 17 ml of 50% sucrose solution (w/v)) was also provided to each cage.

6. A queen pheromones (QMP) strip was provided to each cage for creating environmental conditions as similar as inside the hive.

7. All experimental cages were then placed in an incubator and maintained at 34 ± 2 ˚C with 50 - 55% RH.

3.6.2.4 Survival analysis

1. Dead bees from each cage were counted daily for 30 days. All dead bees were then stored at -21 ◦C for later analysis of infection status.

2. Kaplan-Meier survival curves of all treatment groups were generated by plotting the number of surviving bees against days from the initiation of the experiment.

3. The data were analyzed using a Log-rank test.

3.6.2.5 Infectivity (number of spores per honey bee)

1. The midguts of dead bees collected daily and living bees collected at day 30 p.i. were removed individually and placed in a 0.6 ml microcentrifuge tube containing 100 µl of distilled water.

2. The midgut was then crushed using a sterile plastic pestle.

3. The number of spores was counted using a hemocytometer under a light microscope.

4. The data were represented as means ± SE. The results were analyzed using Kruskal-Wallis test, and Mann-Whitney U test were used to test the differences of median among treatment groups.

3.6.2.6 Infection rate (the percentage of infected bees)

1. The midguts of dead bees collected daily, and alive bees collected at day 30 p.i. were removed individually and placed in a 0.6 ml microcentrifuge tube containing 100 µl of distilled water.

2. The midgut was then crushed using a sterile plastic pestle.

3. Each homogenized sample was determined for *N. ceranae* spores under a light microscope. The results were then calculated as the percentage of infected bees in each treatment group.

4. The data were represented as means ± SE. The results were analyzed using Kruskal-Wallis test, and Mann-Whitney U test were used to test the differences of median among treatment groups.

3.6.2.7 Infection ratio (number of infected ventricular cells to non-infected ventricular cells per a hundred cells)

1. Five bees from each cage were collected on days 3, 6, 10 and 14 p.i., and they were removed their midgut and immediately fixed with Bouin's fluid solution for 24 h.

2. The samples were washed using 70% ethanol until the solution became colorless.

3. Midgut samples were further dehydrated with a series dilution of ethanol; 70% (for 6 h.), 90% (for 6 h.), and 95% (for 6 h. and two times) following the absolute N-butyl for 1 h. and xylene for 1 h.

4. The samples were then transferred to the mixed solution of xylene and paraplast; 2:1, 1:1, and 1:2 at 56 °C for 1 h. per each step before being embedded in a pure melted paraplast at 56 °C for 1 h.

5. After let the samples dried, they were sectioned for 6 µm thickness using a rotary microtome and placed on a glass slide before stained with periodic acid Schiff's reagent (PAS), counterstained with light green.

6. The slides with stained tissues were examined under a light microscope. The infection ratio was then calculated as a proportion of infected cells to non-infected cells over a hundred cells, and the data were represented as a mean percentage ± SE.

7. The results were analyzed using Kruskal-Wallis test, and Mann-Whitney U to test the significant differences among treatment groups.

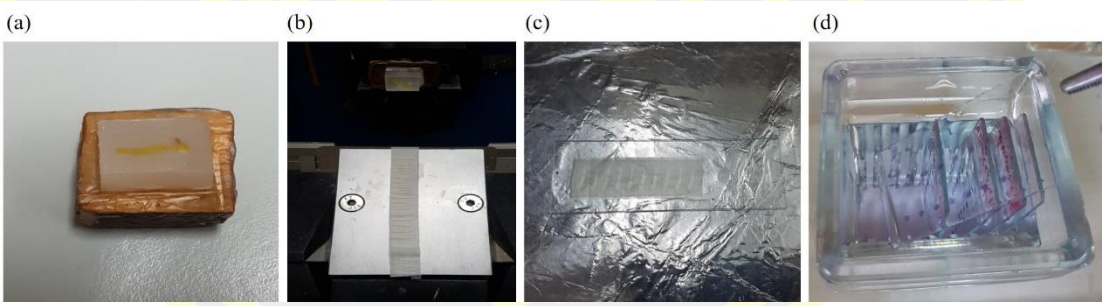

Figure 3.6 Tissue sectioning and staining; the embedded tissue attached to a block (a), sectioned tissue using a rotary microtome (b), the sectioned tissue on a glass slide (c), and tissue staining with periodic acid Schiff's reagent (PAS) and counterstained with light green (d).

3.6.2.8 Trehalose levels in hemolymph

1. Five bees from each cage were collected on days 3, 6, 10 and 14 p.i., and anaesthetized at -21 ˚C for 2 min.

2. The abdomen segment between tergites 3 and 4 was punctured using a sterile small needle to collect 2 µl of honey bee's hemolymph and transferred to a test tube that containing 48 µl of 0.85% sodium chloride.

3. Each tube was added 2.9 ml anthrone reagent and vortexed gently for 30 sec.

4. All tubes were quickly put into boiling water for 15 min before being transferred to cold water for 20 min.

5. The absorbance was measured at 620 nm against a blank using a Shimadzu UV-visible spectrophotometer. The level of trehalose was quantified based on a standard curve generated using various trehalose concentrations (0, 1, 2, 4, 6, and 8 µg per µl).

6. The data were represented as means ± SE. The results were analyzed using the statistical method, Kruskal-Wallis test and Mann-Whitney U to test the significant differences among treatment groups.

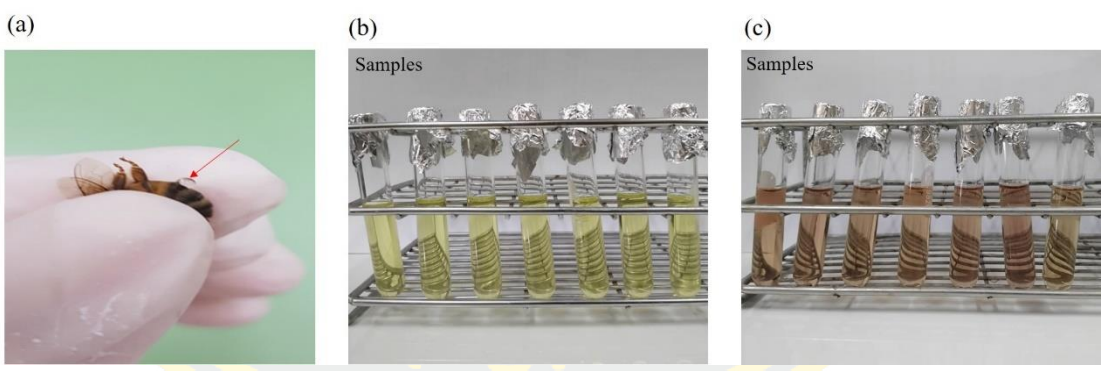

Figure 3.7 Hemolymph collecting and measurement; honey bee hemolymph (a), hemolymph samples after anthrone reagent addition (b), and the samples after boiling for 15 min and cooling for 20 min (c).

3.6.2.9 Sugars in honey bee hemolymph analysis using high-performance liquid chromatography with refractive index (RI) detection (HPLC-RID)

1. Seven bees were collected on day 14 p.i., and anaesthetized at -21 ˚C for 2 min.

2. Two µl of hemolymph was collected from each bee and transfer to a 1.5 ml microcentrifuge tube as pool sample (total = 14 µl) and then kept at -80 °C.

3. The sample was added with 322 µl of 80% acetonitrile (v/v) (total sample volume = 336 µl) and kept on ice.

4. The sampled were centrifuge at 10,000 ×g, 4 °C for 6 min, and 300 µl of supernatant was filtered through a 0.45 µm nylon membrane before transferred to a 2 ml vial glass amber with 250 µl micro glass insert.

5. Twenty µl of each sample was analyzed using an NH_2 column (250 × 4.6 mm, Luna®) with temperature maintain at 40 ºC and the flow rate will be set at 1.0 ml/min.

6. The mobile phase was composed of acetonitrile + water (70 + 30 v/v). The refractive index (RI) detection was operated with polarity +, cell temperature at 35 ºC, and response 1.0 s.

3.6.2.10 Protein content in hypopharyngeal glands

1. Five bees were collected from each cage on days 3, 6, 10 and 14 p.i. and kept frozen at -21 ˚C until further analysis.

2. A pair of hypopharyngeal glands of each bee were removed and placed in a 0.6 µl microcentrifuge tube containing 100 µl of 0.5 M NaOH.

3. Their glands were then crushed, centrifuged at 1,000 g for 2 min, and collected 50 µl of supernatant for further analysis.

4. The protein content of individual bees were determined using the Bradford protein assay (Bradford, 1976). The protein absorbance was measured at 595 nm against a blank reagent (0.5M NaOH) using a Shimadzu UV-visible spectrophotometer.

5. Standard curves were generated using different concentrations of bovine serum albumin (BSA) (0, 250, 500, 750, 1,000, and 1,250 µg per ml) to predict the protein concentration of the sample from absorbance.

6. The data were represented as means ± SE. The results were analyzed using Kruskal-Wallis test and Mann-Whitney U to test the significant differences among treatment groups.

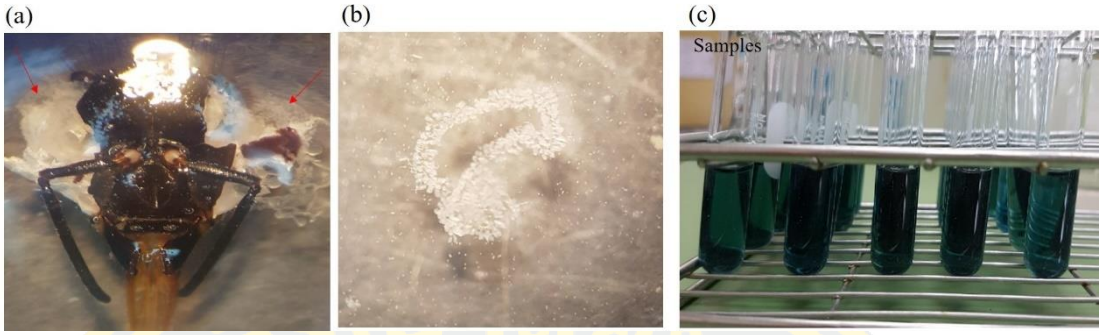

Figure 3.8 Hypopharyngeal gland collecting and protein measurement; honey bee hypopharyngeal glands (a, b), and the color of samples after 6 min of exposure to Coomassie brilliant blue G250 dye (c).

3.6.2.11 Midgut proteolytic enzyme activity

1. Five bees were collected from each cage on days 3, 6, 10, and 14 p.i. Their midgut were each removed and placed in 0.5 µl microcentrifuge tube containing 90 µl of chilled 0.1 M Tris buffer (pH 7.9).

2. Midgut in each tube was crushed using a sterile plastic pestle and then centrifuged at 7,570 g for 6 min.

3. A total 10 µl of the supernatant was transferred to three 1.5 ml microcentrifuge tubes containing 50 µl of the chilled Tris buffer.

4. All tubes were then added 120 µl of 2% azocasein and vortexed gently.

5. Sample blanks, one tube from each sample, were left at room temperature while the other two tubes were incubated at 37 °C for 4 h.

6. After incubation, all tubes were placed on ice and added 600 µl of chilled 10% trichloroacetic acid. Each tube was vortexed well and centrifuged at 7,925 g for 4 min.

7. Then, 700 µl of the supernatant was transferred to a test tube containing 400 µl of 50% ethanol.

8. Each tube was vortexed before reading absorbance at 440 nm against a blank using a Shimadzu UV-visible spectrophotometer.

9. Total midgut proteolytic enzyme activity was calculated from the mean of the replicate readings for each sample and expressed in terms of OD440. The

results were analyzed using Kruskal-Wallis test and Mann-Whitney U to test the significant differences among treatment groups.

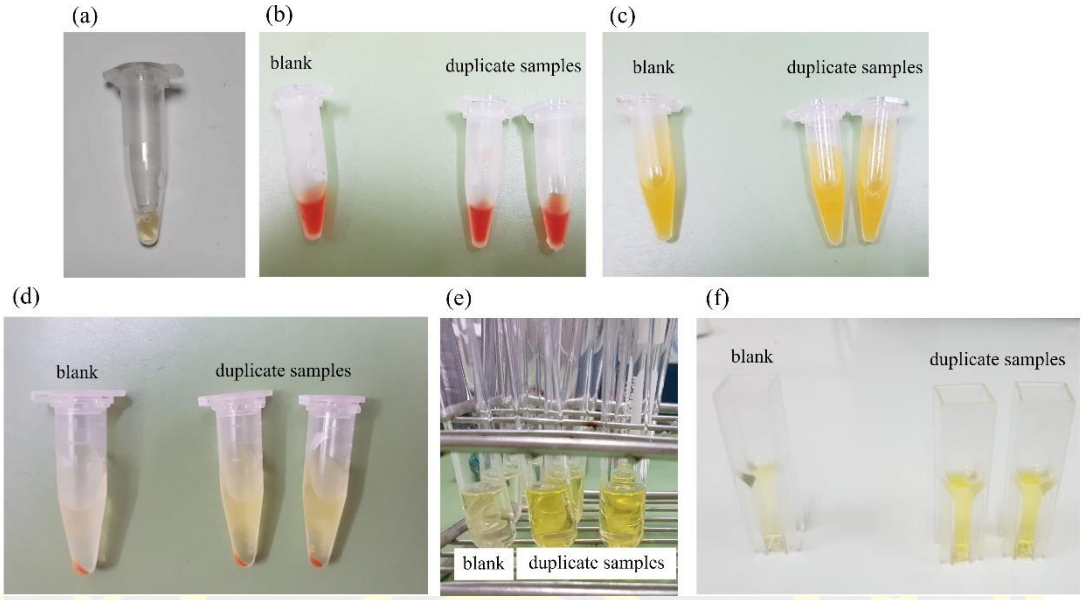

Figure 3.9 Midgut proteolytic enzyme activity measurement; a honey bee midgut in 0.1 M Tris buffer (a), sample supernatant after 2% azocasein addition (b), and samples after chilled 10% trichloroacetic acid addition (c), samples after centrifugation (d), and the sample supernatants mixed with 50% ethanol (e, f).

3.6.3 Experiment III: To investigate the impact of natural products; propolis extract of stingless bee, *Tetrigona apicalis* and chito-oligosaccharide (COS), for the control of *N. ceranae* infection in *A. dorsata* workers compared to those of *A. mellifera* workers.

3.6.3.1 Propolis extract preparation

1. Raw stingless bee propolis was collected from three colonies of *T. apicalis* at an agricultural extension and development center, Chiang Mai Province, Thailand, and stored at -21 °C in a dark container to protect from the light.

2. Propolis was ground into the powder using a mortar and pestle.

3. Ten g of propolis powder were extracted with 100 ml of 80% ethanol at 70 °C and continuously stirred for 1 h.

4. The solvent from the extract was filtered using Whatman paper No.4 to yield a crude ethanolic extract which was assumed as a stock solution

5. The stock solution was then diluted with distilled water to obtain a 50% propolis solution (v/v) and kept in dark at 4 °C until use.

6. A 50% propolis was provided to honey bees by mixing it with pollen (17 ml of 50% propolis solution + 60 g of pollen) and placed in a 1.5 ml microcentrifuge tube which was replaced every two days until the end of the experiment.

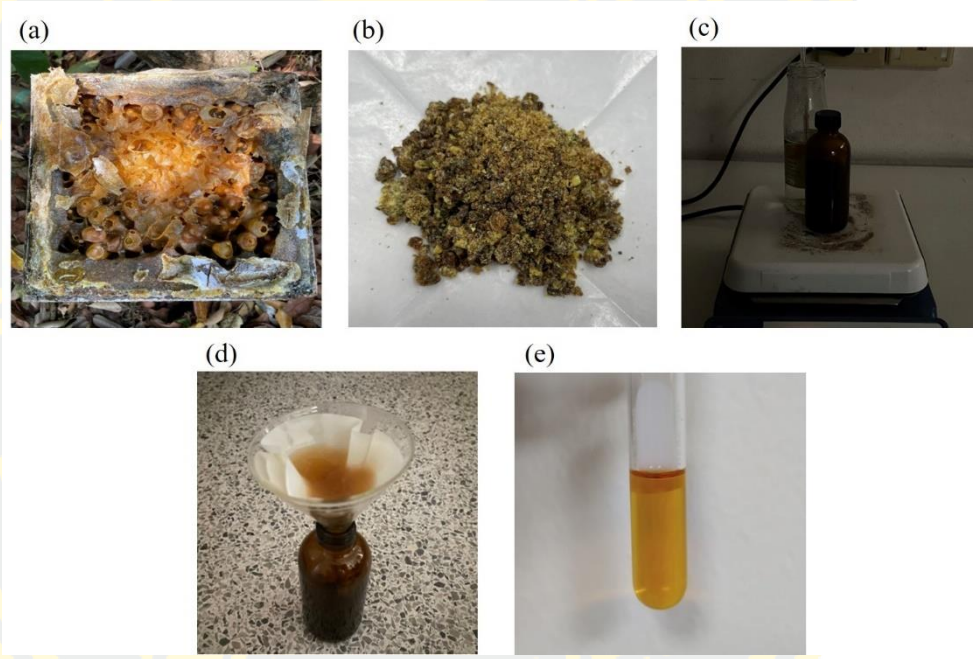

Figure 3.10 Stingless bee propolis extraction; raw propolis in a *T. apicalis* hive located in Chiang Mai Province, Thailand (a), ground propolis (b), propolis extraction using 80% ethanol at 70 °C in a dark room (c), gravity filtration (d), and propolis stock solution (e).

3.6.3.2 Chito-oligosaccharide (COS) solution preparation

1. The stock of 10^4 ppm of COS was prepared by dissolving 0.25 g of COS (6,081 Da) in 5 ml of pure *A. dorsata* honey, and then adjusting the final volume to 25 ml by adding 50% sucrose solution (w/v).

2. The 10^4 ppm COS was then diluted with 50% sterile honey solution in water (v/v) to obtain a 10^2 ppm COS. The same method was used to prepare 0.5 ppm COS.

3. COS was provided to honey bees by mixing it with pollen (17 ml of 0.5 ppm COS + 60 g of pollen) and placing in a 1.5 ml microcentrifuge tube which was replaced every two days until the end of the experiment.

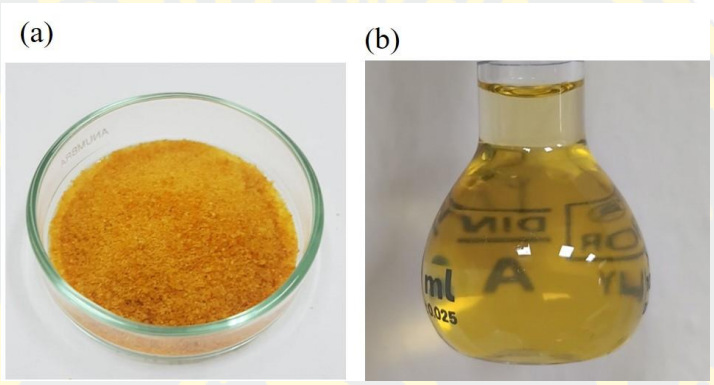

Figure 3.11 Chito-oligosaccharide or COS (6,081 Da), and a stock of 10^4 ppm of COS (b).

3.6.3.3 *Nosema ceranae* spore preparation

1. *Nosema ceranae* spores isolated from *A. mellifera* were obtain from the propagated bees in experiment II.

2. Ten midguts of propagated bees were removed and transferred into 1.5 ml microcentrifuge tubes containing 200 µl of distilled water.

3. Midguts in each tube were then crushed and centrifuged at 6,000 g for 10 min. The white sediment (*N. ceranae* spores) at the bottom of the tube was collected and re-suspended in distilled water. This process was repeated three times.

4. The number of spores was counted under the light microscope using a hemocytometer.

6. Spores were then centrifuged and re-suspended in 50% sucrose solution (w/v) to obtain a concentration of 5×10^5 spores per 2 µl and kept at room temperature for infection.

3.6.3.4 Newly emerged bee preparation

1. Sealed brood frames of *A. dorsata* worker were obtained from healthy *Nosema*-free colonies located in Samut Songkhram Province, Thailand.

2. Sealed brood frames of *A. mellifera* were obtained from three healthy *Nosema*-free colonies located at BeefamBUU, Chon Buri Province, Thailand.

3. The brood frames were then maintained at 34 ± 2 ˚C and 50 - 55% relative humidity (RH) to obtain newly emerged bees (≤ 24 h. old).

3.6.3.5 Experimental infection

1. Newly emerged bees were carefully removed and confined into cages (size: h = 13 cm, r = 5.25 cm) in groups of 50.

2. The experiment was divided into 6 treatment groups with three replicate cages per group.

3. Control bees not treated, bees individually force-fed 2 µl of 50% sucrose solution (w/v) without *N. ceranae* spores and provided with pollen mixed (60 g of pollen mixed with 17 ml of 50% sucrose solution (w/v)) defined as CO.

4. Control bees treated with propolis, bees individually force-fed 2 µl of 50% sucrose solution (w/v) without *N. ceranae* spores and provided with 50% propolis extract in pollen (60 g of pollen mixed with 17 ml of 50% propolis solution (v/v)) defined as CO+PO.

5. Control bees treated with COS, bees individually force-fed 2 µl of 50% sucrose solution (w/v) without *N. ceranae* spores and provided with 0.5 ppm COS in pollen (60 g of pollen mixed with 17 ml of 0.5 ppm COS solution (v/v)) defined as CO+COS.

6. *N. ceranae*-infected bees not treated, bees individually force-fed 2 µl of 50% sucrose solution (w/v) containing 5×10^5 *N. ceranae* spores and provided with pollen mixed defined as NC.

7. Infected bees treated with propolis, bees individually force-fed 2 µl of 50% sucrose solution (w/v) containing 5×10^5 *N. ceranae* spores and provided with 50% propolis extract in pollen defined as NC+PO

8. Infected bees treated with COS, bees individually force-fed 2 µl of 50% sucrose solution (w/v) containing 5×10^5 *N. ceranae* spores and provided with 0.5 ppm COS in pollen defined as NC+COS.

9. Each cage was given 50% sucrose solution (w/v) and distilled water in 2 ml microcentrifuge tubes that were replaced every two days during the experiment. All cages were maintained at 34 ± 2 °C with relative humidity between 50 - 55%.

3.6.3.6 Survival analysis

1. Dead bees from each cage were counted daily for 30 days. All dead bees were then stored at -21 ◦C for later analysis of infection status.

2. Kaplan-Meier survival curves of all treatment groups were generated by plotting the number of surviving bees against days from the initiation of the experiment.

3. The data were analyzed using a Log-rank test.

3.6.3.7 Infectivity (number of spores per honey bee)

1. The midguts of dead bees collected daily and alive bees collected at day 30 post infection (p.i.) were removed individually and placed in a 0.6 ml microcentrifuge tube containing 100 µl of distilled water.

2. The midgut was then crushed using a sterile plastic pestle.

3. The number of spores was counted using a hemocytometer under a light microscope.

4. The data were represented as means ± SE. The results were analyzed using Kruskal-Wallis test, and Mann-Whitney U test were used to test the differences of median among treatment groups.

3.6.3.8 Infection rate (the percentage of infected bees)

1. The midguts of dead bees collected daily, and alive bees collected at day 30 p.i. were removed individually and placed in a 0.6 ml microcentrifuge tube containing 100 µl of distilled water.

2. The midgut was then crushed using a sterile plastic pestle.

3. Each homogenized sample was determined for *N. ceranae* spores under a light microscope. The results were then calculated as the percentage of infected bees in each treatment group.

4. The data were represented as means ± SE. The results were analyzed using Kruskal-Wallis test, and Mann-Whitney U test were used to test the differences of median among treatment groups.

3.6.3.9 Infection ratio (number of infected ventricular cells to non-infected ventricular cells per a hundred cells)

1. Five bees from each cage were collected on days 3, 6, 10 and 14 p.i., and they were removed their midgut and immediately fixed with Bouin's fluid solution for 24 h.

2. The samples were washed using 70% ethanol until the solution became colorless.

3. Midgut samples were further dehydrated with a series dilution of ethanol; 70% (for 6 h.), 90% (for 6 h.), and 95% (for 6 h. and two times) following the absolute N-butyl for 1 h. and xylene for 1 h.

4. The samples were then transferred to the mixed solution of xylene and paraplast; 2:1, 1:1, and 1:2 at 56 °C for 1 h. per each step before being embedded in a pure melted paraplast at 56 °C for 1 h.

5. After let the samples dried, they were sectioned for 6 µm thickness using a rotary microtome and placed on a glass slide before stained with periodic acid Schiff's reagent (PAS), counterstained with light green.

6. The slides with stained tissues were examined under a light microscope. The infection ratio was then calculated as a proportion of infected cells to non-infected cells over a hundred cells, and the data were represented as a mean percentage ± SE.

7. The results were analyzed using Kruskal-Wallis test, and Mann-Whitney U to test the significant differences among treatment groups.

3.6.3.10 Trehalose levels in hemolymph

1. Five bees from each cage were collected on days 3, 6, 10 and 14 p.i., and anaesthetized at -21 ˚C for 2 min.

2. The abdomen segment between tergites 3 and 4 was punctured using a sterile small needle to collect 2 µl of honey bee's hemolymph and transferred to a test tube that containing 48 µl of 0.85% sodium chloride.

3. Each tube was added 2.9 ml anthrone reagent and vortexed gently for 30 sec.

4. All tubes were quickly put into boiling water for 15 min before being transferred to cold water for 20 min.

5. The absorbance was measured at 620 nm against a blank using a Shimadzu UV-visible spectrophotometer. The level of trehalose was quantified based on a standard curve generated using various trehalose concentrations.

6. The data were represented as means ± SE. The results were analyzed using the statistical method, Kruskal-Wallis test and Mann-Whitney U to test the significant differences among treatment groups.

3.6.3.11 Protein content in hypopharyngeal glands

1. Five bees were collected from each cage on days 3, 6, 10 and 14 p.i. and kept frozen at -21 ˚C until further analysis.

2. A pair of hypopharyngeal glands of each bee were removed and placed in a 0.6 µl microcentrifuge tube containing 100 µl of 0.5 M NaOH.

3. Their glands were then crushed, centrifuged at 1,000 g for 2 min, and collected 50 µl of supernatant for further analysis.

4. The protein content of individual bees were determined using the Bradford protein assay (Bradford, 1976). The protein absorbance was measured at 595 nm against a blank reagent (0.5M NaOH) using a Shimadzu UV-visible spectrophotometer.

5. Standard curves were generated using different concentrations of bovine serum albumin (BSA) to predict the protein concentration of the sample from absorbance.

6. The data were represented as means ± SE. The results were analyzed using Kruskal-Wallis test and Mann-Whitney U to test the significant differences among treatment groups.

3.6.3.12 Midgut proteolytic enzyme activity

1. Five bees were collected from each cage on days 3, 6, 10, and 14 p.i. Their midgut were each removed and placed in 0.5 µl microcentrifuge tube containing 90 µl of chilled 0.1 M Tris buffer (pH 7.9).

2. Midgut in each tube was crushed using a sterile plastic pestle and then centrifuged at 7,570 g for 6 min.

3. A total 10 µl of the supernatant was transferred to three 1.5 ml microcentrifuge tubes containing 50 µl of the chilled Tris buffer.

4. All tubes were then added 120 µl of 2% azocasein and vortexed gently.

5. Sample blanks, one tube from each sample, were left at room temperature while the other two tubes were incubated at 37 °C for 4 h.

6. After incubation, all tubes were placed on ice and added 600 µl of chilled 10% trichloroacetic acid. Each tube was vortexed well and centrifuged at 7,925 g for 4 min.

7. Then, 700 µl of the supernatant was transferred to a test tube containing 400 µl of 50% ethanol.

8. Each tube was vortexed before reading absorbance at 440 nm against a blank using a Shimadzu UV-visible spectrophotometer.

9. Total midgut proteolytic enzyme activity was calculated from the mean of the replicate readings for each sample and expressed in terms of OD440. The results were analyzed using Kruskal-Wallis test and Mann-Whitney U to test the significant differences among treatment groups.

3.6.5 Experiment IV: To measure the expression of immune-related genes of *A. dorsata* and *A. mellifera* workers response to the infection of *N. ceranae* and after treating with propolis and COS.

This experimental part using honey bees collected from experiment III for measuring the expression of antimicrobial peptides in *A. dorsata* and *A. mellifera.*

3.6.5.1 RNA extraction and cDNA synthesis

1. Two bees were randomly collected from each cage on day 3, 6, 10 and 14 post infection (p.i.).

2. Each bee was individually stored in a 1.5 ml microcentrifuge tube containing 1 ml of RNAlater solution and immediately stored at -80 °C until analysis.

3. Each bee was crushed in 1 ml of RLT-buffer with 1% beta-mercaptoethanol using a sterile pestle.

4. Total RNA was extracted from 100 µl of bee homogenate using RNeasy Mini Kit, according to the manufacturer's protocol in a QiaCube robot.

5. RNA concentration and quality were measured using the EPOCH microplate spectrophotometer and adjusted to 80 ng/µl using RNAse-free water.

6. First-strand cDNA synthesis was performed by adding 0.8 µl of Oligo-dT to 10 µl of RNA and incubated at 70 °C for 5 min.

7. Then, a master mix containing 3.0 µl of 5x RT-buffer, 0.8 µl of 10 mM dNTP mix, and 0.4 µl M-MLV Revertase was added in a final volume of 15 µl.

8. cDNA Synthesis was carried out at 42 °C for 1 h followed by 72 °C for 15 min, and store at -80 °C till being process qPCR.

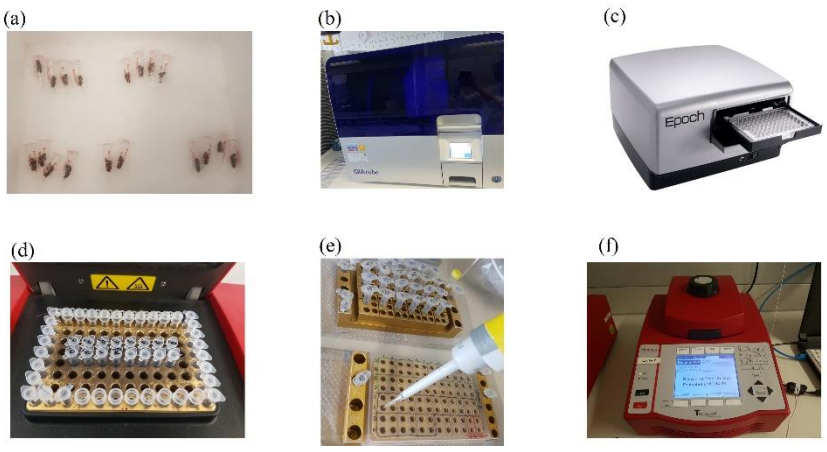

Figure 3.12 RNA extraction and cDNA synthesis; honey bee samples (a), RNA extraction using QiaCube robot (b), RNA concentration measurement using EPOCH microplate spectrophotometer (c), cDNA synthesis (d), PCR amplification (e, f).

3.6.5.2 Real-time quantitative PCR

1. PCR amplification was performed in a 10 µl reaction mixture using 1 µl of ten times diluted cDNA, 5 µl of SYBR Sensi Mix, 3.6 µl of nuclease-free water and 0.2 µl of each specific primer (10 µM) that showed in Table 1.

2. PCR reactions were carried out in 96-well microtiter plates. The amplification was programmed as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s for β-actin, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 53 °C for 30 s and 72 °C for 60 s for antimicrobial peptide-encoding genes of *A. dorsata*, and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 60 s for antimicrobial peptide-encoding genes of *A. mellifera*.

3. Fluorescence measurements were taken repeatedly during the 95 °C step. The amplification results were expressed as the quantification cycle (Ct) value, which represented the number of cycles needed to generate a fluorescent signal greater than a predefined threshold.

4. Relative quantification was calculated by using Ct value for the target gene subtracted from the mean of the reference genes, β-actin to generate ΔCt values for each sample.

5. To generate ΔΔCt values, the ΔCt values were then normalized with the lowest expression level (highest ΔCt value) of the target for that sampling period.

6. To compare expression levels across treatments using the qPCR results, data were transformed using the $2^{-\Delta\Delta Ct}$ method, and the results were expressed as n-fold differences relative to the calibrator. The data were analyzed using a Kruskal-Wallis test, and Mann-Whitney U test.

Table 3.1 qPCR primers used for the amplification of genes related to innate immunity in *A. dordata* and *A. mellifera.*

Primer name	Sequence	Target gene amplified	Honey bee species
ApidaecinF_Ador	5′-TTTTACCTTAGCAATTCTTGTTA-3′	apidectin	*A. dorsata*
ApidaecinR_Ador	5′-GCAGGTTGAGTAGGCGGATCT-3′		
defensinF_Ador	5′-TGCGCTGCTAACTGTCTCAG -3′	defensin	*A. dorsata*
defensinR_Ador	5′-AATTGACTTAACCGAAACG-3′		
HymenoptaecinF_Ador	5′-CTTTTCTGCGCTATTGCATA-3′	hymenoptaecin	*A. dorsata*
HymenoptaecinR_Ador	5′-GCCTCTCCTGTCATTCCATT-3′		
ApidNT-F	5′-TTTTGCCTTAGCAATTCTTGTTG-3′	apidectin	*A. mellifera*
ApidNT-R	5′-GTAGGTCGAGTAGGCGGATCT-3′		
Defensin-F	5′-TGCGCTGCTAACTGTCTCAG-3′	defensin	*A. mellifera*
Defensin-R	5′-AATGGCACTTAACCGAAACG-3′		
Hymenopt-F	5′-CTCTTCTGTGCCGTTGCATA-3′	hymenoptecin	*A. mellifera*
Hymenopt-R	5′-GCGTCTCCTGTCATTCCATT-3′		
Am-Actin2-qF	5′- CGT GCC GAT AGT ATT CTT G-3′	β -actin (reference housekeeping gene)	*A. dorsata* and *A. mellifera*
Am-Actin2-qB	5′- CTT CGT CAC CAA CAT AGG-3′		

CHAPTER 4
RESULTS

4.1 Experiment I: To examine the virulence of *Nosema ceranae* isolated from four honey bee species of Thailand; *Apis cerana*, *A. dorsata*, *A. florea*, and *A.mellifera* in the giant honey bee, *A. dorsata* workers.

4.1.1 Identification of *Nosema* spores

Nosema spores isolated from *Apis cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* workers were identified using PCR to confirm the species. The result showed that *Nosema* spores isolated from four honey bee species, *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellfera* are *N. ceranae* (Figure 4.1).

Figure 4.1 PCR products of *Nosema* spores isolated from *A. cerana*, *A dorsata*, *A. florea*, and *A. mellifera* workers; a 146 bp is *N. ceranae* and a 200 bp = a marker.

4.1.2 Infection rate

No *N. ceranae* spores were found in any bees from non-infected groups or control bees throughout the experiment. Moreover, the infection rate of NC-AC, NC-AD, NC-AF, and NC-AM after dosed with 1×10^2 and 1×10^3 spores per bee were 0.00 ± 0.00%. The infection rate of NC-AC, NC-AD, NC-AF, and NC-AM after dosed with 1×10^4 spores per bee were 12.70 ± 0.09%, 43.70 ± 0.18%, 37.15 ± 1.28%, and 42.98 ± 1.65%, respectively, with the infection rate of NC-AD was significantly higher than NC-AF and NC-AC but not NC-AM ($\chi^2 = 14.73$, $df = 3$, $p = 0.0020$, Figure 4.2a). The infection rate of NC-AC, NC-AD, NC-AF, and NC-AM after dosed with 1×10^5 spores per bee were 14.84 ± 0.24%, 72.73 ± 2.03%, 60.00 ± 0.00%, and 73.08 ± 1.72%, respectively, with the infection rate of NC-AD and NC-AM were significantly higher than NC-AF and NC-AC ($\chi^2 = 16.07$, $df = 3$, $p = 0.0009$, Figure 4.2b). The infection rate of NC-AC (42.31 ± 1.72%) after dosed with 5×10^5 spores per bee was significantly lower than NC-AD, NC-AF, and NC-AM which were 100.00 ± 0.00% of infection ($\chi^2 = 10.71$, $df = 3$, $p = 0.0003$, Figure 4.2c). However, the infection rate of NC-AC, NC-AD, NC-AF, and NC-AM were 100.00 ± 0.00% when dosed with 1×10^6 spores per bee (Figure 4.2d).

4.1.3 Infectious dose (ID_{50} and ID_{100})

The mean infectious doses required to infect 50% (ID_{50}) of *A. dorsata* workers were approximately 550,000 spores for *N. ceranae* isolated from *A. cerana* (NC-AC), 54,000 spores for *N. ceranae* isolated from *A. florea* (NC-AF), and 20,000 spores for *N. ceranae* isolated from *A. dorsata* (NC-AD) and *A. mellifera* (NC-AM). All worker bees of *A. dorsata* in NC-AD, NC-AF, and NC-AM become infected, therefore the infectious dose required to infect 100% (ID_{100}) of *A. dorsata* workers was approximately 500,000 spores for *N. ceranae* isolated from *A. dorsata, A. florea,* and *A. mellifera*. The ID_{100} of *A. dorsata* workers was approximately 1,000,000 spores for *N. ceranae* spores isolated from *A. mellifera* (Figure 4.3).

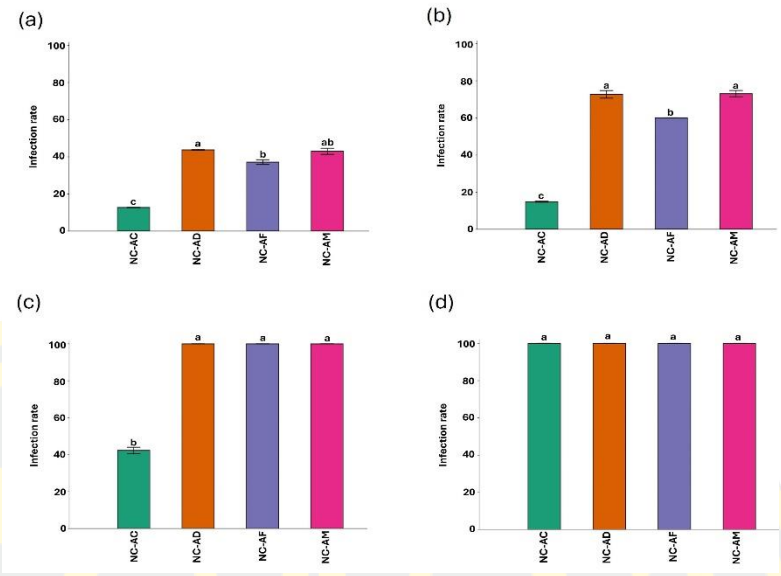

Figure 4.2 Bar chart of infection rate (number of infected bees per a hundred bees) of *A. dorsata* after dosed with *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* (defined as NC-AC, NC-AD, NC-AF, and NC-AM, respectively) at the dosage of 1×10^4 (a), 1×10^5 (b), 5×10^5 (c), and 1×10^6 (d) spores per bee. The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $\chi^2 = 14.73$, $df = 3$, $p = 0.0020$, $\chi^2 = 16.07$, $df = 3$, $p = 0.0009$, $\chi^2 = 10.71$, $df = 3$, $p = 0.0003$, and $\chi^2 = 0.00$, $df = 3$, $p = 1.000$ for graphs a - d, respectively).

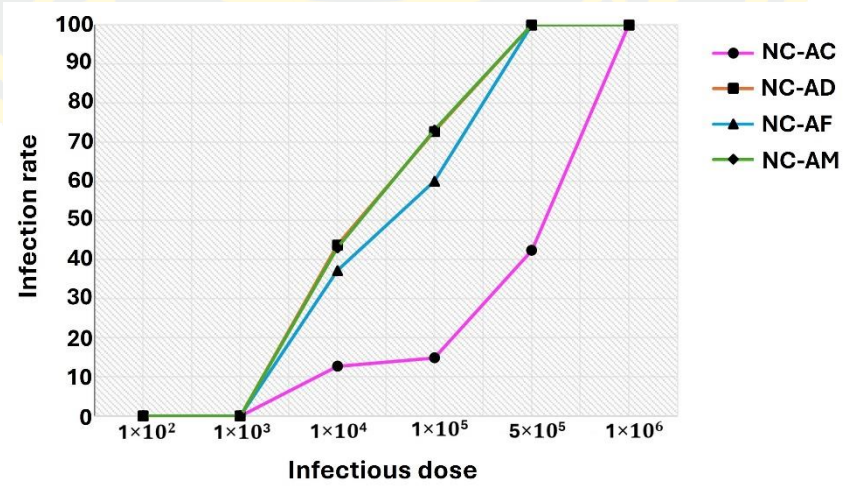

Figure 4.3 The infectious dose of *A. dorsata* after dosed with *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. forea*, and *A. mellifera* (defined as NC-AC, NC-AD, NC-AF, and NC-AM, respectively) at the dosage of 1×10^2, 1×10^3, 1×10^4, 1×10^5, 5×10^5, and 1×10^6 spores per bee.

4.1.4 Infectivity (number of spores per honey bee)

No *N. ceranae* spores were found in non-infected bees and NC-AC, AN-AD, NC-AF, and NC-AM dosed with 1×10^2 and 1×10^3 spores per bee throughout the experiment. There was no significance different in infectivity among *N. ceranae*-infected groups; NC-AC, AN-AD, NC-AF, and NC-AM when fed *N. ceranae* at the concentration 5×10^5 ($\chi^2 = 5.47$, $df = 3$, $p = 0.1399$, Figure 4.4c) and 1×10^6 ($\chi^2 = 7.21$, $df = 3$, $p = 0.0656$, Figure 4.4d) spores per bee. *Nosema ceranae* isolated from *A. cerana* (NC-AC) was significantly lower than other infected bee groups, NC-AD, NC-AF, and NC-AM when fed *N. ceranae* at the concentration of 1×10^4 ($\chi^2 = 12.75$, $df = 3$, $p = 0.0004$, Figure 4.4a), and 1×10^5 ($\chi^2 = 20.61$, $df = 3$, $p < 0.0001$, Figure 4.4b).

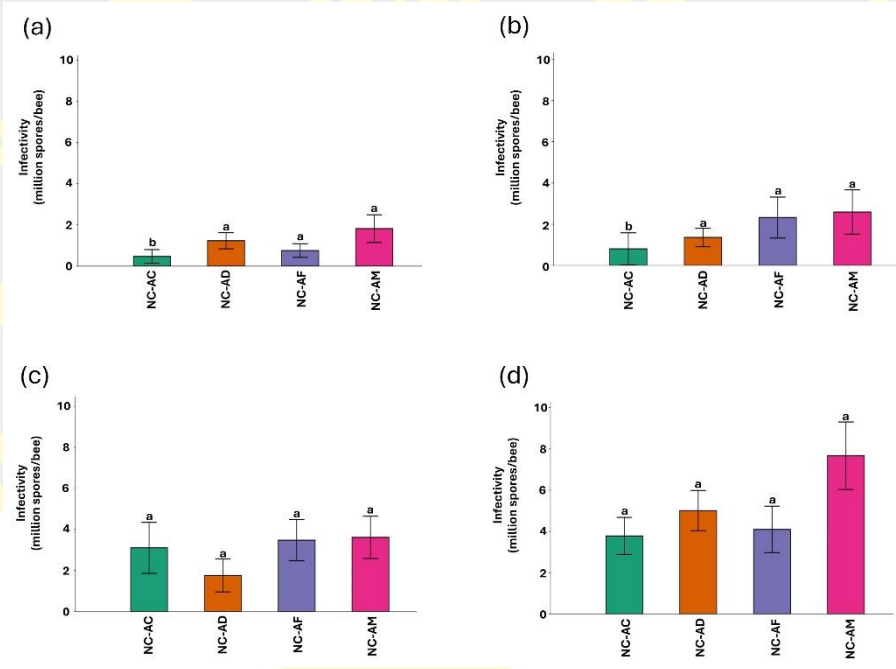

Figure 4.4 Bar chart of infectivity (number of *N. ceranae* spores per bee) of *A. dorsata* dosed with *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* (defined as NC-AC, NC-AD, NC-AF, and NC-AM, respectively) at the dosage of 1×10^4 (a), 1×10^5 (b), 5×10^5 (c), and 1×10^6 (d) spores per bee. The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $\chi^2 = 12.75$, $df = 3$, $p = 0.0004$, $\chi^2 = 20.61$, $df = 3$, $p < 0.0001$, $\chi^2 = 5.47$, $df = 3$, $p = 0.1399$, and $\chi^2 = 7.21$, $df = 3$, $p = 0.0656$ for graphs a - d, respectively).

4.1.5 Survival analysis

The survival probabilities of all *N. ceranae*-infected groups were significantly lower than non-infected group (control bees) when dosed with all concentrations of *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* (Figure 4.5). There was no significant difference among infected bee groups; NC-AC, AN-AD, NC-AF, and NC-AM when fed *N. ceranae* at the concentration of 1×10^2 ($\chi^2 = 13.49$, $df = 4$, $p = 0.0066$, Figure 4.5a), 1×10^3 ($F = 14.59$, $df = 4$, $p = 0.0004$, Figure 4.5b), 1×10^4 ($F = 6.51$, $df = 4$, $p = 0.0076$, Figure 4.5c), 1×10^5 ($\chi^2 = 11.54$, $df = 4$, $p = 0.0176$, Figure 4.5d), and 1×10^6 ($\chi^2 = 14.39$, $df = 4$, $p = 0.0061$, Figure 4.5f) spores per bee. Moreover, the survival probability of bees dosed with 5×10^5 *N. ceranae* isolated from *A. mellifera* (NC-AM) was significantly lower than NC-AC, NC-AD, and NC-AF ($\chi^2 = 20.15$, $df = 4$, $p = 0.0004$, Figure 4.5e).

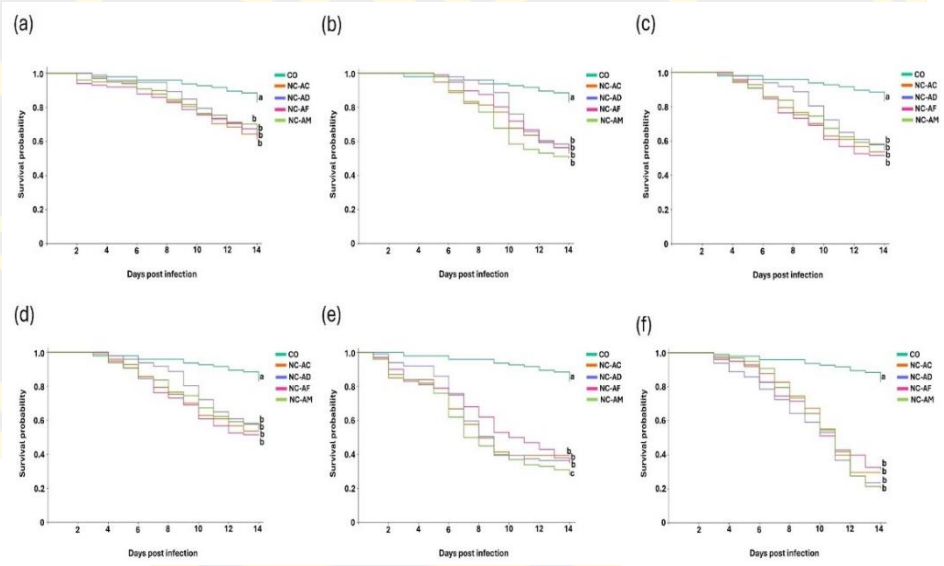

Figure 4.5 Survival probability of *A. dorsata* after dosed with *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* (defined as NC-AC, NC-AD, NC-AF, and NC-AM, respectively) at the dosage of 1×10^2 (a), 1×10^3 (b), 1×10^4 (c), 1×10^5 (d), 5×10^5 (e), and 1×10^6 (f) spores per bee. The different letters indicate significantly different from one another (Log-rank test; $\chi^2 = 12.10$, $df = 4$, $p = 0.0005$, $\chi^2 = 16.37$, $df = 4$, $p < 0.0001$, $\chi^2 = 21.10$, $df = 4$, $p < 0.0001$, $\chi^2 = 41.59$, $df = 4$, $p < 0.0001$, $\chi^2 = 46.39$, $df = 4$, $p < 0.0001$, and $\chi^2 = 59.70$, $df = 4$, $p < 0.0001$ for graphs a-f, respectively).

4.1.6 Food consumption

Sucrose consumption of *A. dorsata* workers in NC-AC, NC-AD, NC-AF, and NC-AM were not significantly different from control bees (CO) when dosed with spores at concentration of 1×10^2, 1×10^3, 1×10^4, 1×10^5, 5×10^5, and 1×10^6 spores per bee ($F = 0.50$, $df = 4$, $p = 0.7363$, $F = 1.16$, $df = 4$, $p = 0.3492$, $F = 0.14$, $df = 4$, $p = 0.9641$, $F = 0.76$, $df = 4$, $p = 0.5582$, $F = 0.61$, $df = 4$, $p = 0.6596$, and $F = 0.16$, $df = 4$, $p = 0.9588$, respectively, Figure 4.6).

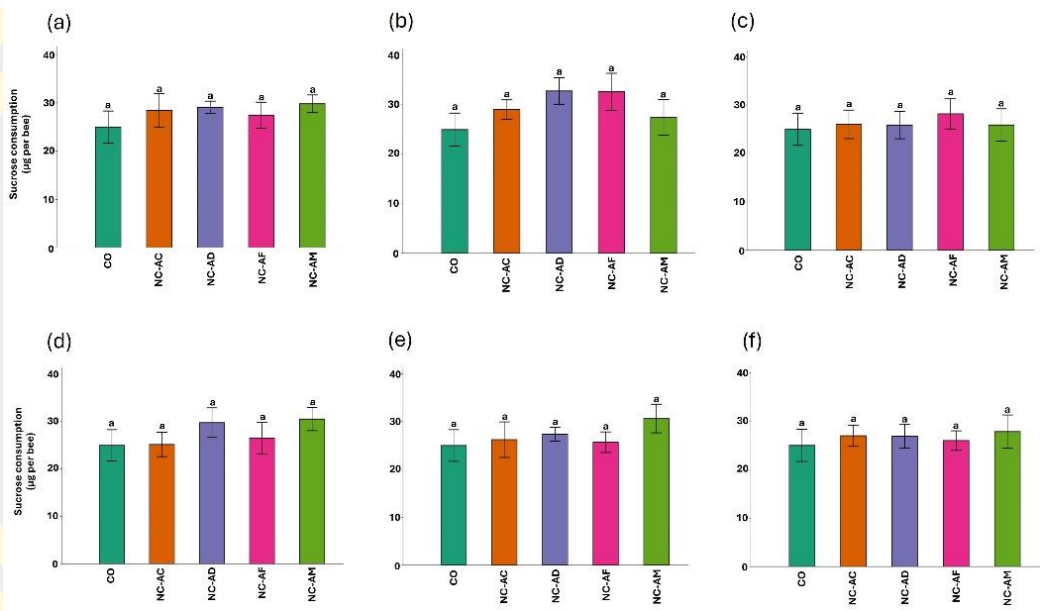

Figure 4.6 Sucrose consumption (mg per bee) of *A. dorsata* dosed with *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* (defined as NC-AC, NC-AD, NC-AF, and NC-AM, respectively) at the dosage of 1×10^2 (a), 1×10^3 (b), 1×10^4 (c), 1×10^5 (d), 5×10^5 (e), and 1×10^6 (f) spores per bee, and control bees (CO). The different letters indicate significantly different from one another (One-way ANOVA; $F = 1.16$, $df = 4$, $p = 0.3492$, $F = 1.16$, $df = 4$, $p = 0.3492$, $F = 0.14$, $df = 4$, $p = 0.9641$, $F = 0.76$, $df = 4$, $p = 0.5582$, $F = 0.61$, $df = 4$, $p = 0.6596$, and $F = 0.16$, $df = 4$, $p = 0.9588$ for graphs a-f, respectively).

Honey bees in NC-AC, NC-AD, NC-AF, and NC-AM when dosed with *N. ceranae* at 1×10^2, 1×10^3, and 1×10^4 spores per bee, consumed water which were not significantly different from CO ($F = 1.63$, $df = 4$, $p = 0.1915$, $F = 0.34$, $df = 4$, $p = 0.8482$, and $F = 3.83$, $df = 4$, $p = 0.1254$, respectively. Figure 4.7a-c). However, there were significantly different in the mean of water consumption among NC-AC, NC-AD, NC-AF, NC-AM, and CO when dosed *N. ceranae* at 1×10^5, 5×10^5, and 1×10^6 spores per bee ($F = 4.09$, $df = 4$, $p = 0.0092$, $F = 4.36$, $df = 4$, $p = 0.0067$, and $F = 2.87$, $df = 4$, $p = 0.0399$, respectively, Figure 4.7d-f).

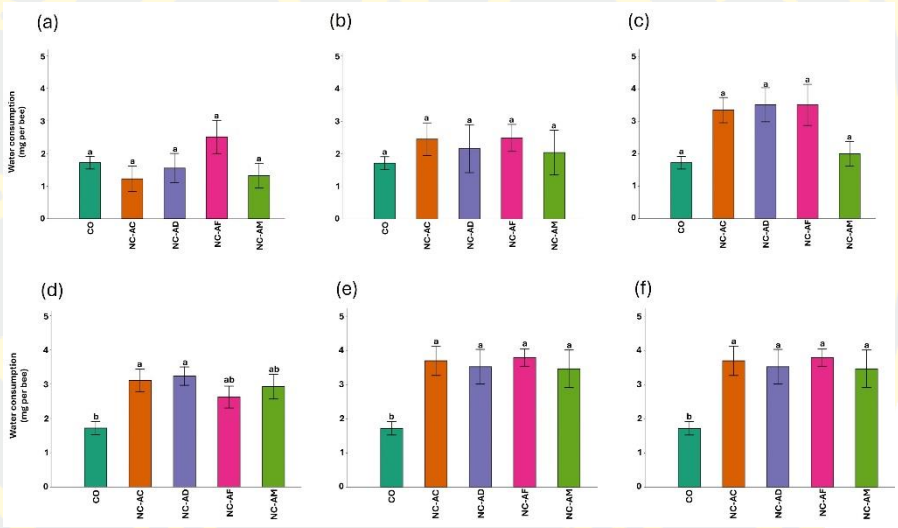

Figure 4.7 Water consumption (mg per bee) of *A. dorsata* dosed with *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* (defined as NC-AC, NC-AD, NC-AF, and NC-AM, respectively) at the dosage of 1×10^2 (a), 1×10^3 (b), 1×10^4 (c), 1×10^5 (d), 5×10^5 (e), and 1×10^6 (f) spores per bee, and control bees (CO). The different letters indicate significantly different from one another (One-way ANOVA; $F = 1.63$, $df = 4$, $p = 0.1915$, $F = 0.34$, $df = 4$, $p = 0.8482$, $F = 3.83$, $df = 4$, $p = 0.1254$, $F = 4.09$, $df = 4$, $p = 0.0092$, $F = 4.36$, $df = 4$, $p = 0.0067$, and $F = 2.87$, $df = 4$, $p = 0.0399$ for graphs a-f, respectively).

Pollen consumption of NC-AC, NC-AD, NC-AF, and NC-AM when dosed with *N. ceranae* at 1×10^2, 1×10^3, and 1×10^4 spores per bee were not significantly different from CO ($F = 1.10$, $df = 4$, $p = 0.3747$, $F = 0.8846$, $df = 4$, $p = 0.4850$, and $F = 1.90$, $df = 4$, $p = 0.1367$, respectively, Figure 4.8a-c). Moreover, there were significantly different in the pollen consumption among NC-AC, NC-AD, NC-AF, NC-AM, and CO when dosed *N. ceranae* at 1×10^5, 5×10^5, and 1×10^6 spores per bee ($\chi^2 = 14.51$, $df = 4$, $p = 0.0058$, $\chi^2 = 12.8$, $df = 4$, $p = 0.0122$, and $F = 2.80$, $df = 4$, $p = 0.0437$, respectively, Figure 4.8d-f).

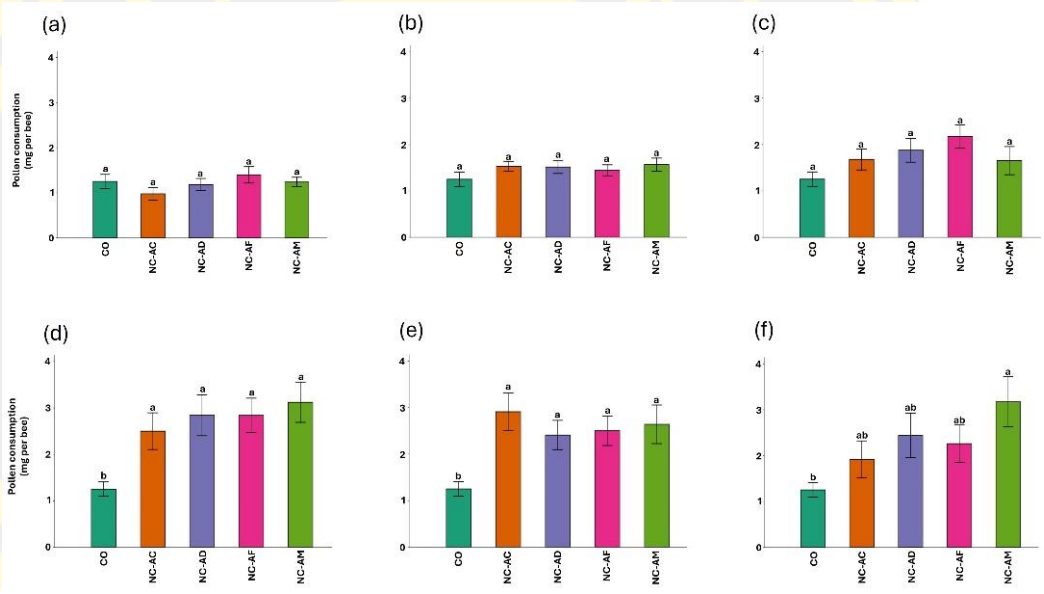

Figure 4.8 Pollen consumption (mg per bee) of *A. dorsata* dosed with *N. ceranae* isolated from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* (defined as NC-AC, NC-AD, NC-AF, and NC-AM, respectively) at the dosage of 1×10^2 (a), 1×10^3 (b), 1×10^4 (c), 1×10^5 (d), 5×10^5 (e), and 1×10^6 (f) spores per bee, and control bees (CO). The different letters indicate significantly different from one another (One-way ANOVA and Kruskal-Wallis test; $F = 1.10$, $df = 4$, $p = 0.3747$, $F = 0.8846$, $df = 4$, $p = 0.4850$, $F = 1.90$, $df = 4$, $p = 0.1367$, $\chi^2 = 14.51$, $df = 4$, $p = 0.0058$, $\chi^2 = 12.8$, $df = 4$, $p = 0.0122$, and $F = 2.80$, $df = 4$, $p = 0.0437$ for graphs a-f, respectively).

4.2 Experiment II: To investigate the effect of *N. ceranae* isolated from *A. mellifera* on energetic stress of *A. dorsata* workers using a dosage of 5×10^5 spores per bee (a dosage that results in 100% infection (ID_{100})).

4.2.1 Survival analysis

The survival probability of *N. ceranae* infected bees were significantly lower than control bees ($F = 778.8$, $df = 1$, $p < 0.0001$, Figure 4.9). The percentage of survivals were 53.00 ± 1.34% and 8.00 ± 0.89% for bees in CO and NC groups, respectively.

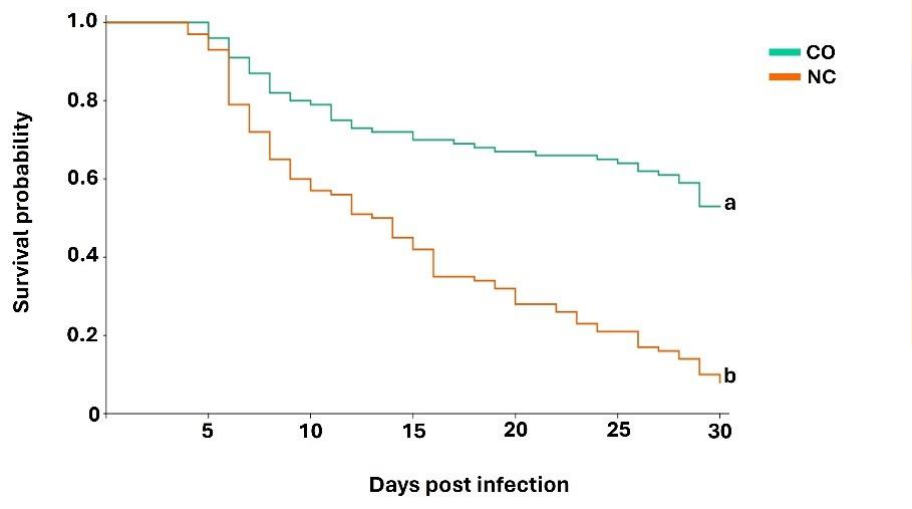

Figure 4.9 Survival probability for 30 days of *A. dorsata* after dosed with *N. ceranae* isolated from *A. mellifera* (defined as NC) at the dosage of 5×10^5 spores per bee compared to control bees (defined as CO). The different letters indicate significantly different from one another (Log-rank test; $\chi^2 = 49.19$, $df = 1$, $p < 0.0001$).

4.2.2 Infectivity (number of spores per honey bee)

The infectivity of *N. ceranae* infected bees (NC) were significantly higher compared to control bees ($\chi^2 = 228.80$, $df = 1$, $p < 0.0001$, Figure 4.10). No *N.*

ceranae spores were found in control bees throughout the experiment. The number of *N. ceranae* spores of NC group was $5.96 \pm 1.00 \times 10^6$ spores per bee.

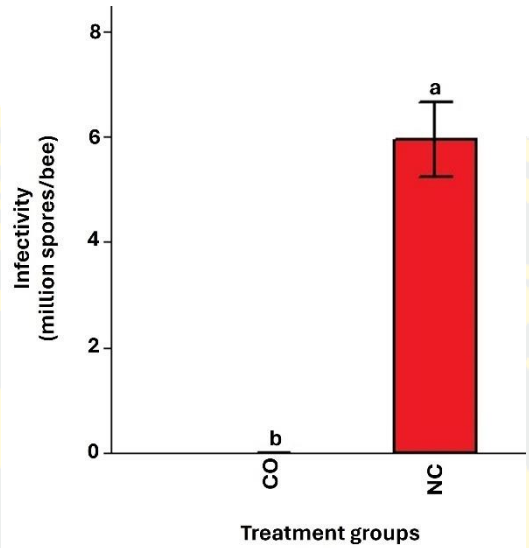

Figure 4.10 Bar chart of infectivity (number of *N. ceranae* spores per bee) at 30 days p.i. of *A. dorsata* workers after dosed with *N. ceranae* isolated from *A. mellifera* (defined as NC) at the dosage of 5×10^5 spores per bee compared to control bees (defined as CO). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $\chi^2 = 228.80$, $df = 1$, $p < 0.0001$).

4.2.3 Infection rate (the percentage of infected bees)

No *N. ceranaae* spores were found in any bees from control groups throughout the experiment. The infection rate of *A. dorsata* dosed with 5×10^5 *N. ceranae* spores per bee was a 100% and was significantly higher compared to control ($\chi^2 = 3.86$, $df = 1$, $p = 0.0254$, Figure 4.11).

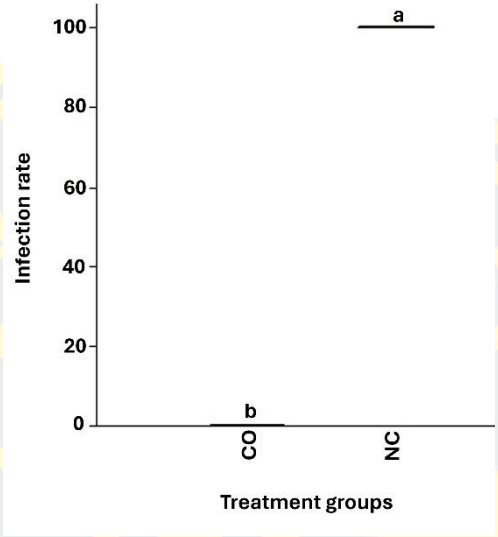

Figure 4.11 Box plots of infection rate (number of infected bees per a hundred bees) of *A. dorsata* after dosed with *N. ceranae* isolated from *A. mellifera* (defined as NC) at the dosage of 5×10^5 spores per bee compared to control bees (defined as CO). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $\chi^2 = 3.86$, $df = 1$, $p = 0.0254$).

4.2.4 Infection ratio (number of infected ventricular cells to non-infected ventricular cells per a hundred cells)

No infected cell was found in control bees throughout the experiment. The infection ratio of bees fed with *N. ceranae* at the dosage of 5×10^5 spores per bee were 13.87 ± 0.48%, 21.00 ± 2.23%, 47.00 ± 2.28%, and 68.87 ± 0.75% on day 3, 6, 10, and 14 p.i., respectively (Figure 4.12). The infection ratio was significant increased when increasing time infection ($\chi^2 = 33.84$, $df = 7$, $p < 0.0001$).

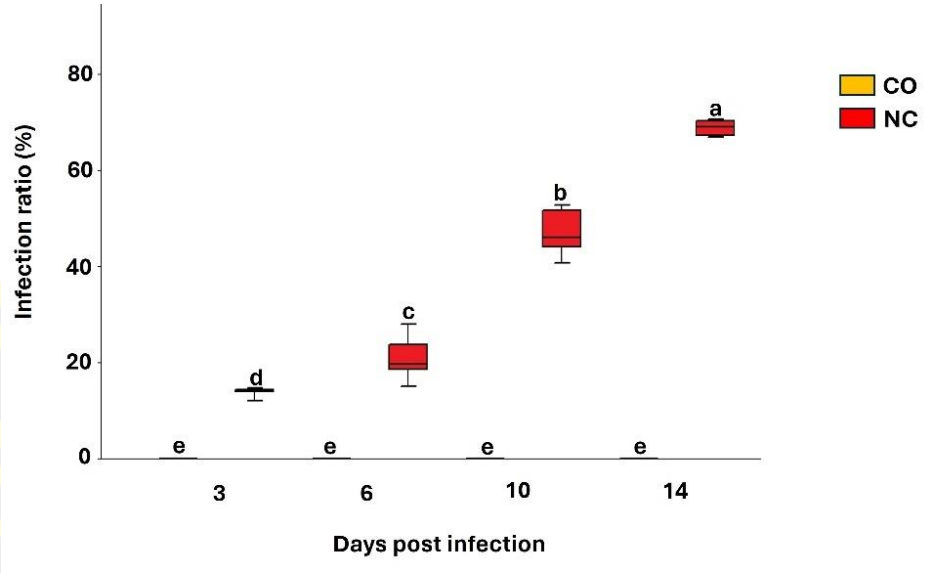

Figure 4.12 Box plots of infection ratio (number of infected cells to non-infected cells over a hundred cells) of *A. dorsata* after dosed with *N. ceranae* isolated from *A. mellifera* (defined as NC) at the dosage of 5×10^5 spores per bee on days 3, 6, 10, and 14 p.i. compared to control (defined as CO). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $\chi^2 = 33.84$, $df = 7$, $p < 0.0001$).

The ventricular cells of bees from the control group showed no *Nosema* spores throughout the experiment (Figure 4.13, 4.15, 4.17, and 4.19). The *Nosema*-infected bees at 3 days p.i. showed the distribution of *N. ceranae* spores in the cytoplasm of some ventricular cells (Figure 4.14). However, only few ventricular cells were found *Nosema* spores infection. On day 6 p.i. (Figure 4.16), more *Nosema* spores were observed in the ventricular cells. The apical cytoplasm were enlarged and filled with a large number of *Nosema* spores. The mature spores were ovoid shape, and surrounded by a wall (Figure 4.18 and 4.20). *Nosema* spores also could be seen in the gut lumen. Moreover, the distribution of *N. ceranae* spores on day 14 appeared to be more heavily infected.

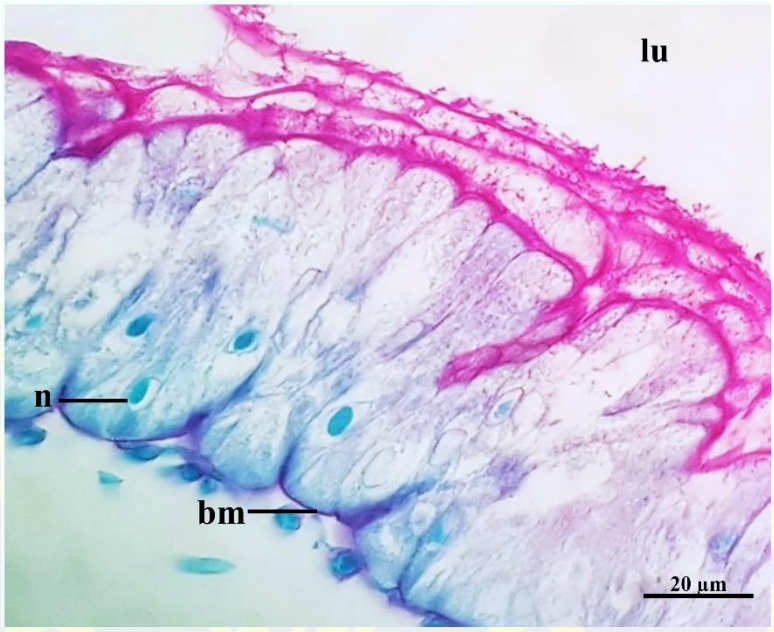

Figure 4.13 Light micrograph of midgut ventricular cells of *A. dorsata* worker from control bee (CO) on day 3 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

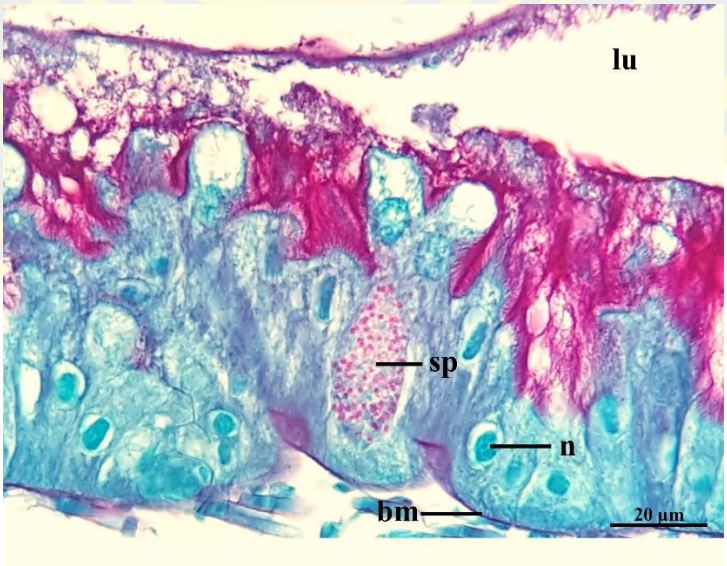

Figure 4.14 Light micrograph of midgut ventricular cells of *A. dorsata* worker from *N. ceranae*-infected bee (NC) on day 3 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

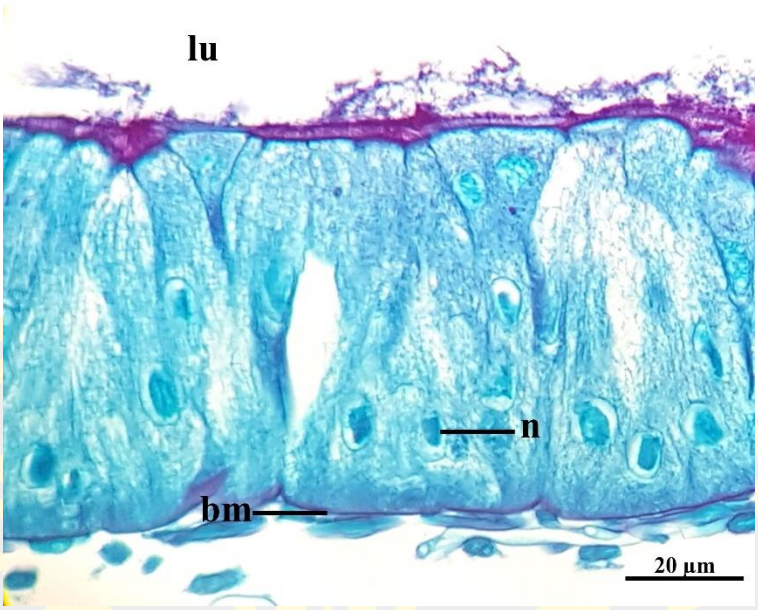

Figure 4.15 Light micrograph of midgut ventricular cells of *A. dorsata* worker from control bee (CO) on day 6 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

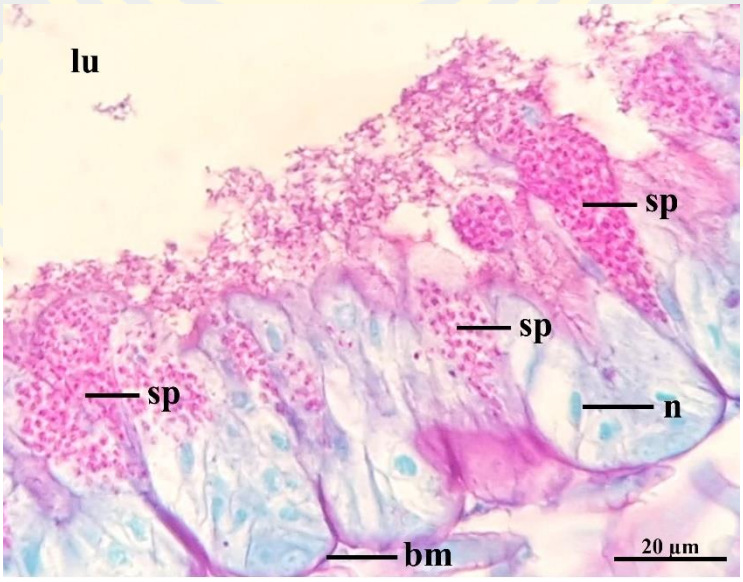

Figure 4.16 Light micrograph of midgut ventricular cells of *A. dorsata* worker from *N. ceranae*-infected bee (NC) on day 6 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

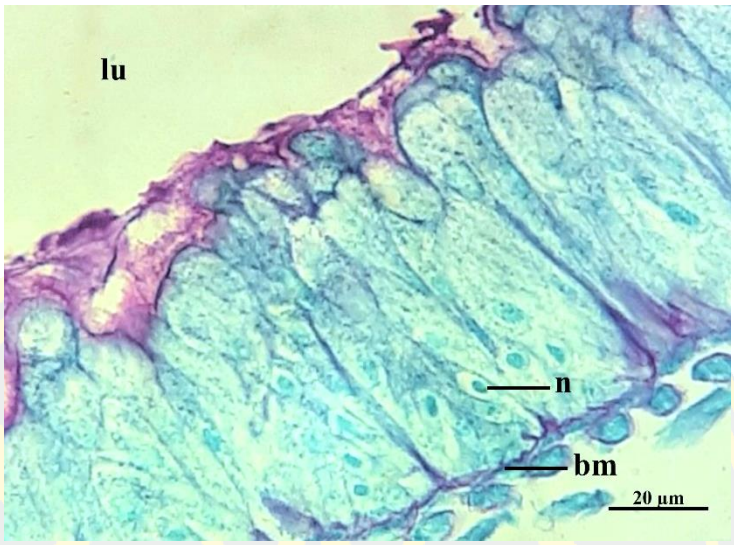

Figure 4.17 Light micrograph of midgut ventricular cells of *A. dorsata* worker from control bee (CO) on day 10 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

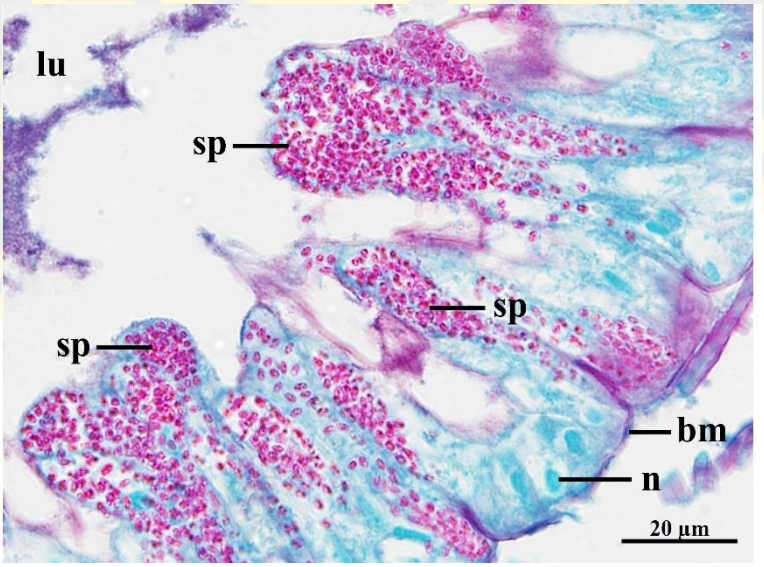

Figure 4.18 Light micrograph of midgut ventricular cells of *A. dorsata* worker from *N. ceranae*-infected bee (NC) on day 10 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

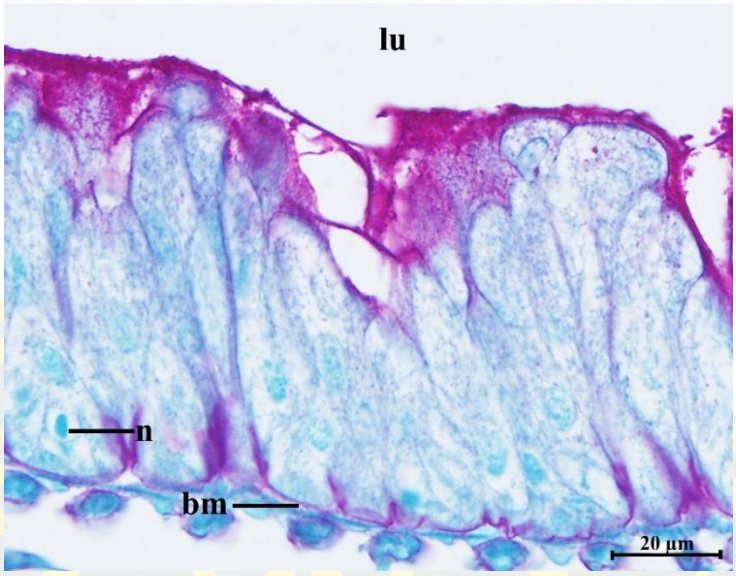

Figure 4.19 Light micrograph of midgut ventricular cells of *A. dorsata* worker from control bee (CO) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

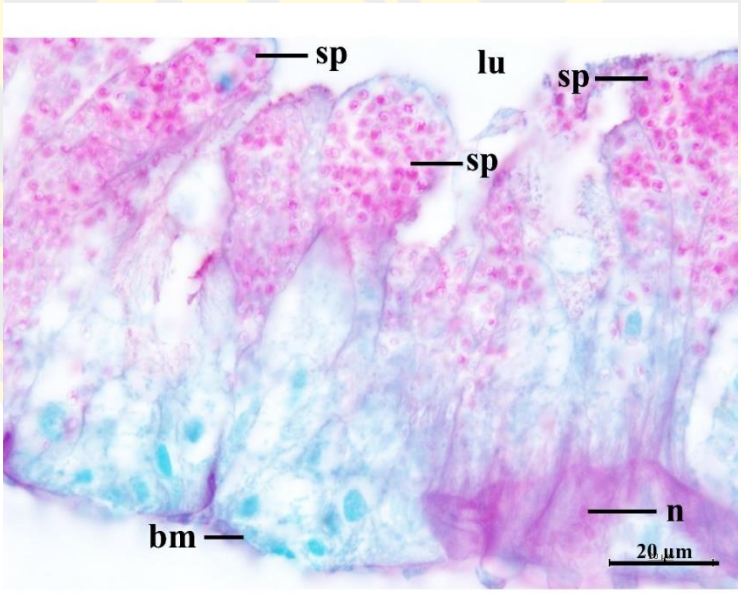

Figure 4.20 Light micrograph of midgut ventricular cells of *A. dorsata* worker from *N. ceranae*-infected bee (NC) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

4.2.5 Trehalose level in hemolymph

The trehalose levels in hemolymph of *A. dorsata* infected with 5×10^5 *N. ceranae* (NC) were significantly lower than those of control bees (CO) on day 3, 6, 10, and 14 p.i. ($\chi^2 = 81.70$, $df = 7$, $p < 0.0001$, Figure 4.21). Trehalose level of NC trend to decrease with increasing time of infection. However, there was no significant difference in trehalose levels of NC at each time detections.

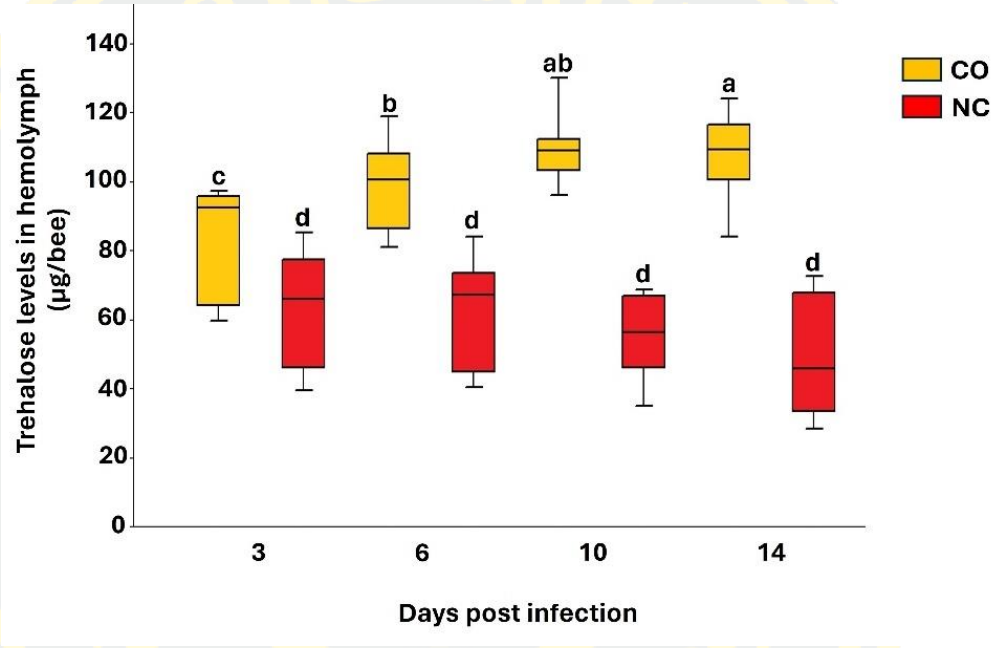

Figure 4.21 Box plots of trehalose levels in bee's hemolymph (µg per bee) of *A. dorsata* after dosed with *N. ceranae* isolated from *A. mellifera* (defined as NC) at the dosage of 5×10^5 spores per bee on days 3, 6, 10, and 14 p.i. compared to control bees (defined as CO). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $\chi^2 = 81.70$, $df = 7$, $p < 0.0001$).

4.2.6 Trehalose levels in hemolymph: high-performance liquid chromatography with refractive index (RI) detection (HPLC-RID)

The pool hemolymph samples collected from control bees (CO) and *N. ceranae* -infected bees (NC, Table 4.2) on day 14 p.i. were analyzed for fructose, glucose, and trehalose in the hemolymph. The trehalose, glucose, and fructose of pool

hemolymph samples of CO were 0.92, 0.24, and 0.52 mg/ml, where as trehalose, glucose, and fructose of pool hemolymph samples of NC were 0.60, 0.04, and 0.15 mg/ml.

Table 4.1 Peak area (nRIU*s) of standard mixture of fructose, glucose, and trehalose at different concentrations.

sugars	**concentration (mg/ml)**				
	0.25	**0.5**	**1.0**	**2.0**	**2.5**
fructose	40,127.60	92,133.00	171,428.00	263,787.00	422,136.00
glucose	36,664.60	88,445.20	183,135.00	261,621.00	414,672.00
trehalose	58,292.10	62,918.60	187,898.00	266,590.00	417,578.00

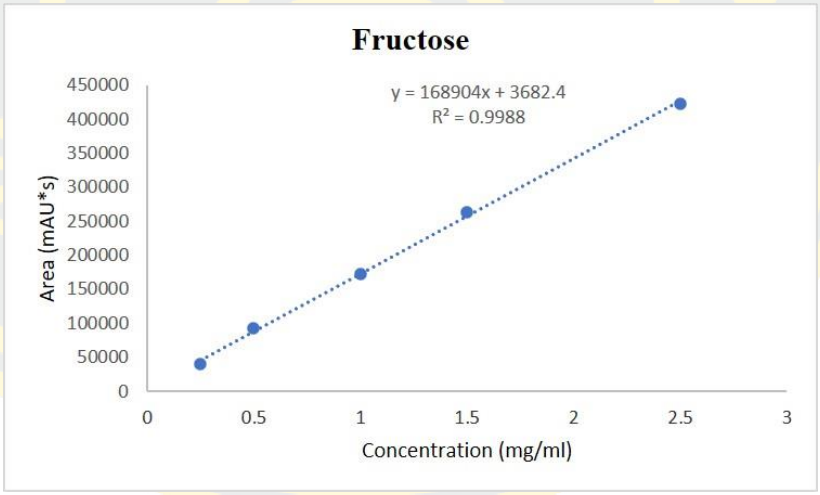

Figure 4.22 The calibration curve of fructose at concentration of 0.25, 0.5, 1.5, and 2.5 mg/ml.

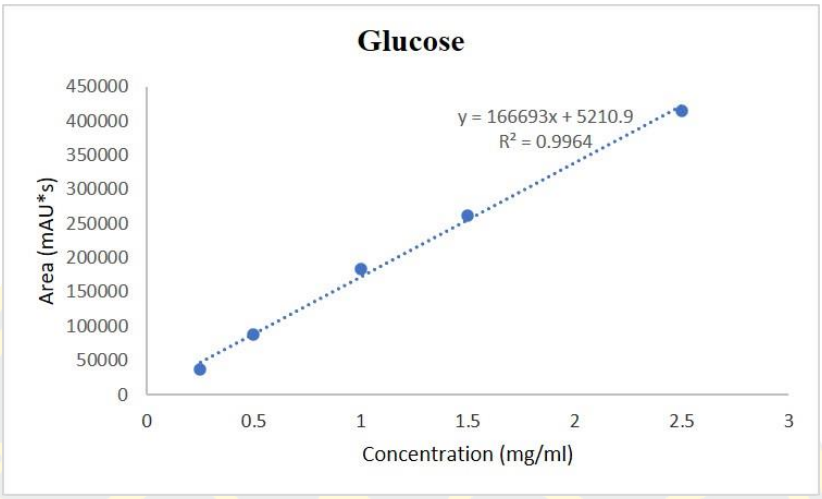

Figure 4.23 The calibration curve of glucose at concentration of 0.25, 0.5, 1.5, and 2.5 mg/ml.

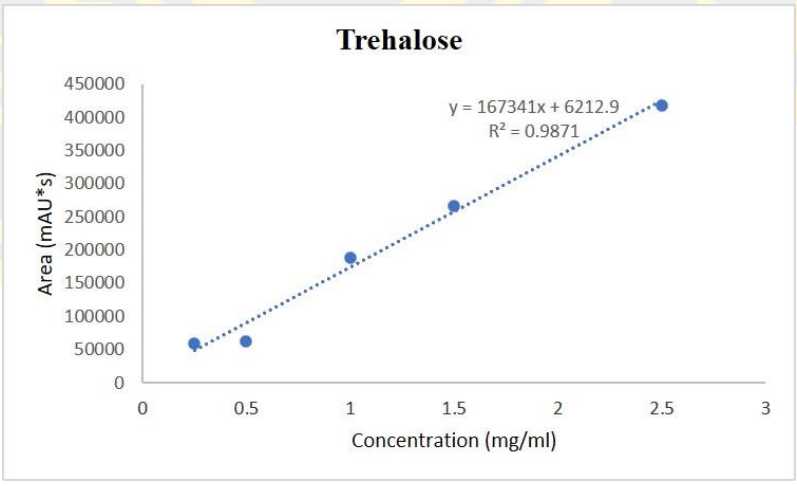

Figure 2.24 The calibration curve of trehalose at concentration of 0.25, 0.5, 1.5, and 2.5 mg/ml.

Table 4.2 The concentration of fructose, glucose, and trehalose in honey bee hemolymph.

treatments	sugars	peak area (nRIU*s)	concentration (mg/ml)
CO	fructose	91,717.10	0.52
	glucose	51,657.50	0.24
	trehalose	181,963.00	0.92
NC	fructose	28,428.40	0.15
	glucose	16,606.70	0.04
	trehalose	118,834.00	0.60

4.2.7 Hypopharyngeal gland protein content

The hypopharyngeal gland protein content of *A. dorsata* infected with 5×10^5 *N. ceranae* (NC) were significantly lower than those of control bees (CO) on day 6, 10, and 14 p.i. ($X^2 = 55.59$, $df = 7$, $p < 0.0001$, Figure 4.25). Protein content of NC trend to decrease with increasing time of infection, and there was significant differences among time point detections.

4.2.8 Midgut proteolytic enzyme activity

The midgut proteolytic enzyme activity of *A. dorsata* infected with 5×10^5 *N. ceranae* (NC) were significantly lower than those of control bees (CO) on day 6, 10, and 14 p.i. ($X^2 = 74.27$, $df = 7$, $p < 0.0001$, Figure 4.26). midgut proteolytic enzyme of CO and NC trend to decrease with increasing time of infection, and there was significant differences among time point detections.

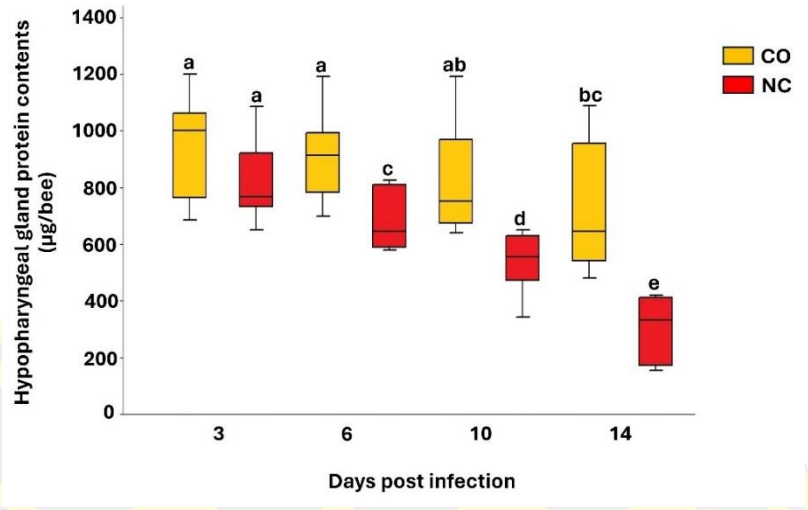

Figure 4.25 Box plots of protein contents in hypopharyngeal glands (µg per bee) of *A. dorsata* after dosed with *N. ceranae* isolated from *A. mellifera* (defined as NC) at the dosage of 5×10^5 spores per bee on day 3, 6, 10, and 14 p.i. compared to control bees (defined as CO). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $X^2 = 55.59$, $df = 7$, $p < 0.0001$).

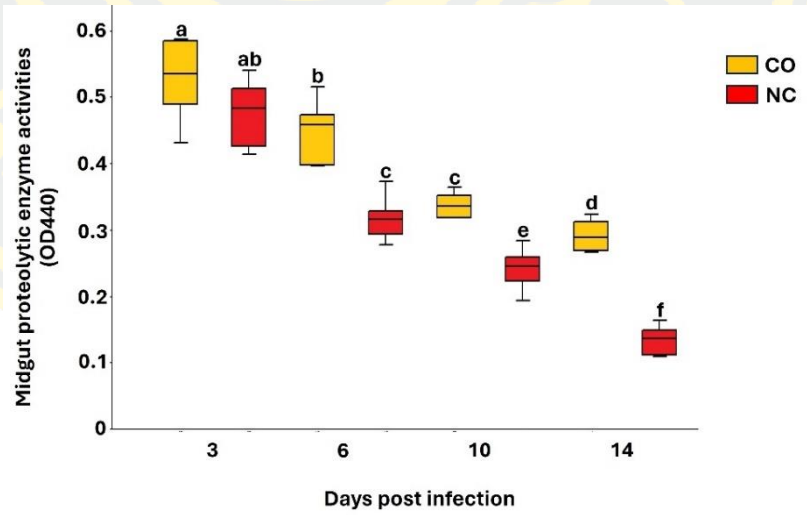

Figure 4.26 Box plots of total midgut proteolytic enzyme activity (OD440) of *A. dorsata* after dosed with *N. ceranae* isolated from *A. mellifera* (defined as NC) at the dosage of 5×10^5 spores per bee on days 3, 6, 10, and 14 p.i. compared to control bees (defined as CO). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $X^2 = 74.27$, $df = 7$, $p < 0.0001$).

4.3 Experiment III: To investigate the impact of natural products; propolis extract of stingless bee, *Tetrigona apicalis* and chito-oligosaccharide (COS), for the control of *N. ceranae* infection in *A. dorsata* workers compared to those of *A. mellifera* workers.

4.3.1 Survival analysis

The survival probabilities of *A. dorsata* in NC+PO and NC+COS were higher than NC, but lower than control bees; CO, CO+PO, and CO+COS (Figure 4.27). The percent survivals of CO, CO+PO, and CO+COS (47.50 ± 4.03%, 55.50 ± 6.75%, and 58.50 ± 9.36%, respectively) were not significantly different from each other ($\chi^2 = 19.50$, $df = 5$, $p = 0.0015$). The percent survivals of NC+PO and NC+COS (7.00 ± 3.00% and 7.00 ± 2.38%, respectively) were higher than NC which no bee survived.

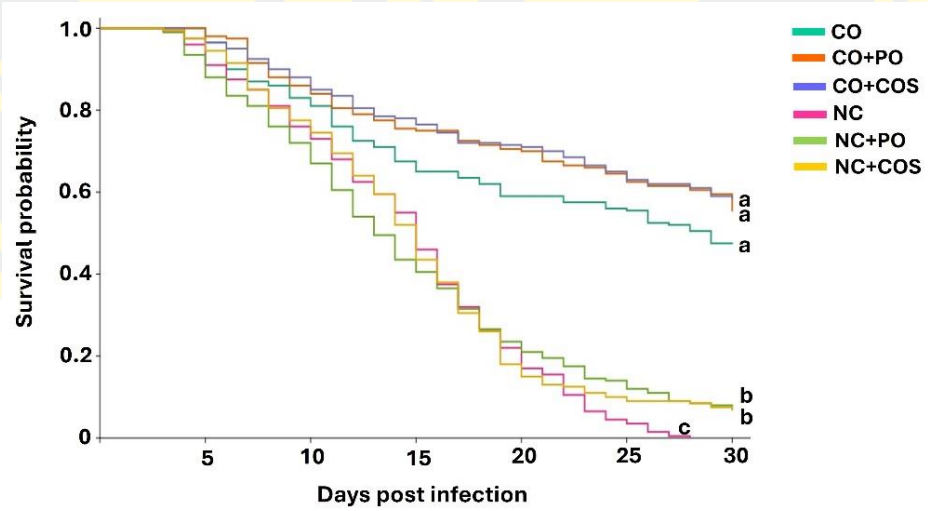

Figure 4.27 The survival probability for 30 days of control bees not treated (CO), control bees treated with 50% propolis (CO+PO), control bees treated with 0.5 ppm COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with 50% propolis (NC+PO), and infected bees treated with 0.5 ppm COS (NC+COS) (Log-rank test; $\chi^2 = 3.26$, $df = 5$, $p = 0.0712$).

For *A. mellifera*, the survival probabilities of NC+PO and NC+COS were higher than NC, but lower than control bees; CO, CO+PO, and CO+COS (Figure 4.28). The percent survivals of CO (58.00 ± 5.37%) were significantly higher than CO+PO (45.00 ± 2.24%), but not different from CO+COS (59.00 ± 0.45%) (χ^2 = 26.00, *df* = 5, *p* = 0.0001). The percent survivals of NC+PO and NC+COS (11.00 ± 2.24% and 13.00 ± 1.34%, respectively) were higher than NC which no bee survived on day 30 p.i.

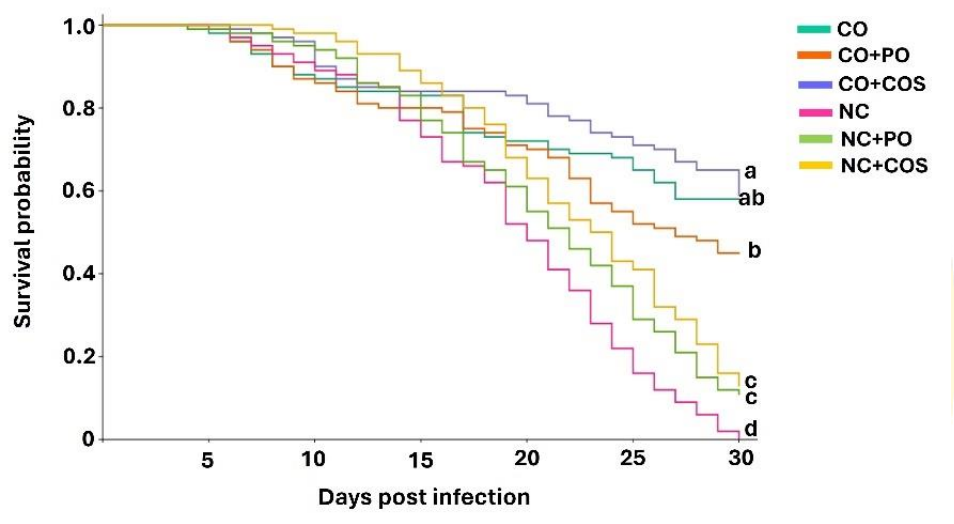

Figure 4.28 The survival probability for 30 days of control bees not treated (CO), control bees treated with 50% propolis (CO+PO), control bees treated with 0.5 ppm COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with 50% propolis (NC+PO), and infected bees treated with 0.5 ppm COS (NC+COS) (Log-rank test; χ^2 = 2.74, *df* = 5, *p* = 0.0976).

4.3.2 Infectivity (number of spores per honey bee)

There were no *N. ceranae* spores in non-infected bee groups (CO, CO+PO, and CO+COS) throughout the experiment in both *Apis* species (Figure 4.29). The overall infectivities of infected *A. mellifera* were significantly higher than those of infected *A. dorsata* (χ^2 = 1,259.00, *df* = 11, *p* < 0.0001). The highest infectivity was found in *A. mellifera* in NC which was 23.17 ± 2.45 × 10^6 spores per bee, following *A. mellifera* in NC+PO and NC+COS which were 15.72 ± 1.94 × 10^6 and 14.37 ± 2.04 × 10^6 spores per bee, respectively. The infectivity of *A. dorsata* in NC, NC+PO,

and NC+COS were 6.24 ± 0.49 × 10^6, 4.12 ± 0.34 × 10^6, and 3.68 ± 0.32 × 10^6 spores per bee, respectively. The infectivity of both honey bee species in NC+PO and NC+COS was significantly lower than that of NC (χ^2 = 1,259.00, *df* = 11, $p < 0.0001$).

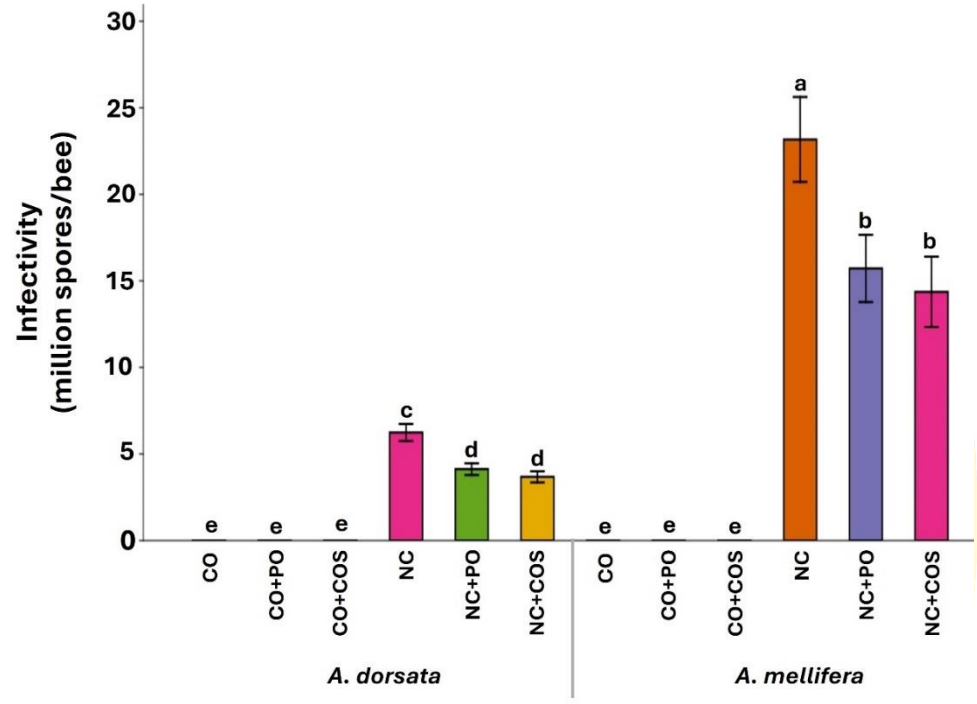

Figure 4.29 Bar chart of infectivity (number of *N. ceranae* spores per bee) for 30 days p.i. of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with 50% propolis (CO+PO), control bees treated with 0.5 ppm COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with 50% propolis (NC+PO), and infected bees treated with 0.5 ppm COS (NC+COS). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; χ^2 = 1,259.00, *df* = 11, $p < 0.0001$).

4.3.3 Infection rate

The infection rate of *A. dorsata* in NC+PO and NC+COS were 88.50 ± 0.50% and 87.00 ± 0.58%, respectively (Figure 4.30). These rates were significantly lower than *A. dorsata* in NC, and *A. mellifera* in NC, NC+PO, and NC+COS which had 100.00 ± 0.00% of infection (χ^2 = 39.25, *df* = 11, $p < 0.0001$). There were no *N.*

ceranae spores in non-infected bee groups (CO, CO+PO, and CO+COS) throughout the experiment.

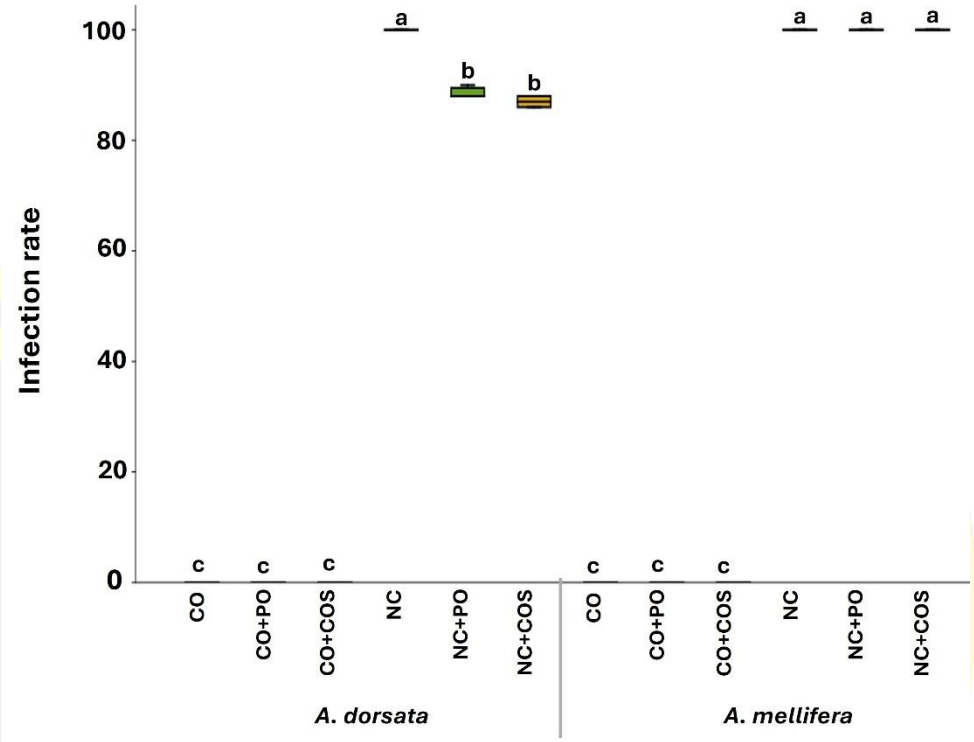

Figure 4.30 Box plots of infection rate (the percentage of infected bees) for 30 days p.i. of *A. dorsata* and *A. melllifera*; control bees not treated (CO), control bees treated with 50% propolis (CO+PO), control bees treated with 0.5 ppm COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with 50% propolis (NC+PO), and infected bees treated with 0.5 ppm COS (NC+COS). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $\chi^2 = 39.25$, $df = 11$, $p < 0.0001$).

4.3.1.4 Infection ratio (number of infected ventricular cells to non-infected ventricular cells per a hundred cells)

The overall infection ratio of infected *A. mellifera* were significantly higher than those of infected *A. dorsata* ($\chi^2 = 216.20$, $df = 11$, $p < 0.0001$). The infection ratio of *A. dorsata* in NC, NC+PO, and NC+COS were 61.87 ± 0.98%, 37.85 ± 1.39%, and

36.80 ± 1.24%, respectively (Figure 4.31), while the infection ratio of *A. mellifera* in NC, NC+PO, and NC+COS were 68.09 ± 1.62%, 46.74 ± 1.62%, and 48.53 ± 2.93%, respectively. The infection ratios of both honey bee species in NC+PO and NC+COS were significantly lower than those of bees in NC. There was no *N. ceranae* spores in the ventricular cells of non-infected groups (CO, CO+PO, and CO+COS).

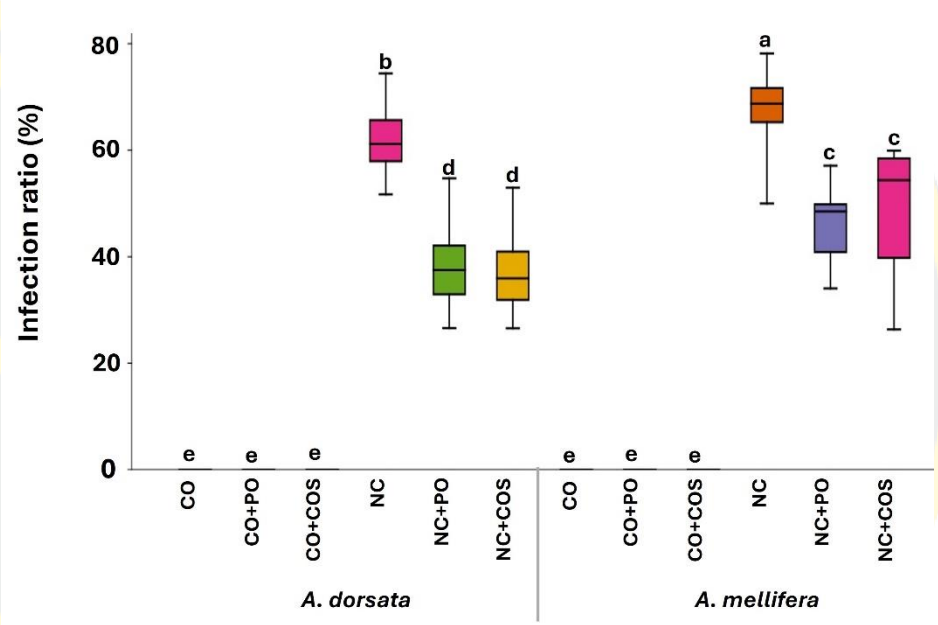

Figure 4.31 Box plots of infection ratio (number of infected cells to non-infected cells per a hundred cells) on day 14 p.i. of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with 50% propolis (CO+PO), control bees treated with 0.5 ppm COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with 50% propolis (NC+PO), and infected bees treated with 0.5 ppm COS (NC+COS). The different letters above the vertical bars indicate significantly different from one another (Kruskal-Wallis test; $\chi^2 = 216.20$, $df = 11$, $p < 0.0001$).

The midgut ventricular cells of *A. dorsata* in control bee not treated (CO, Figure 4.32), control bee treated with propolis (CO+PO, Figure 4.33), and control bee treated with COS (CO+COS, Figure 4.34) on day 14 p.i. showed no *Nosema* spore inside the ventricular cells of the honey bee, while *N. ceranae* spores were found in infected bee groups (NC, NC+PO, and NC+COS, Figures 4.35-4.37). Heavy infection was found in infected bee not treated (NC, Figure 4.35), and the spores were distributed throughout the cytoplasm of midgut ventricular cells. *Nosema* spores were found from the apical part to the bottom of the cell cytoplasm, close to the basement membrane. The swollen cytoplasm and the degeneration of the apical membrane could be seen. However, lower infection was found in infected bee treated with propolis (NC+PO, Figure 4.36) and infected bee treated with COS (NC+COS, Figure 4.37).

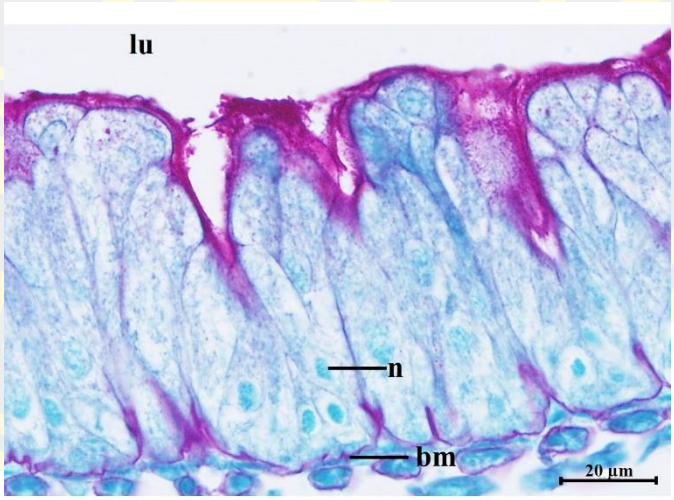

Figure 4.32 Light micrograph of midgut ventricular cells of *A. dorsata* worker from control bee not treated (CO) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

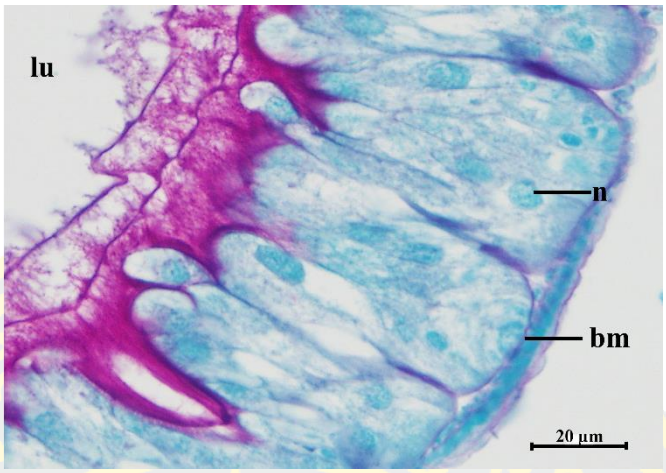

Figure 4.33 Light micrograph of midgut ventricular cells of *A. dorsata* worker from control bee treated with 50% propolis extract (CO+PO) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

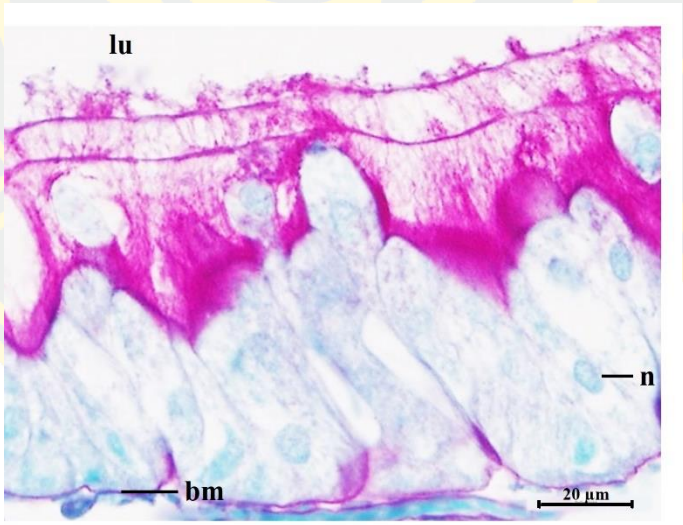

Figure 4.34 Light micrograph of midgut ventricular cells of *A. dorsata* worker from control bee treated with 0.5 ppm COS (CO+COS) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

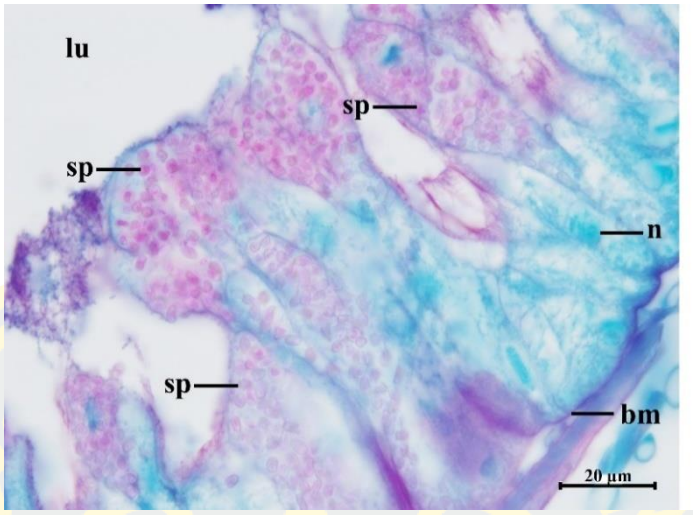

Figure 4.35 Light micrograph of infected midgut ventricular cells of *A. dorsata* worker from *N. ceranae*-infected bee not treated (NC) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

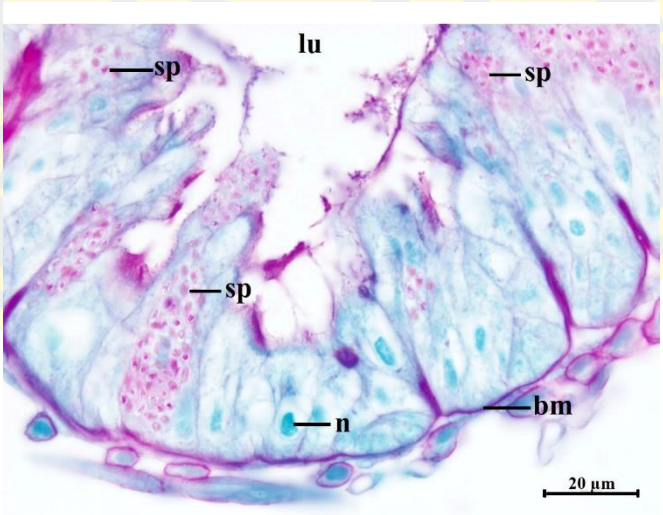

Figure 4.36 Light micrograph of infected midgut ventricular cells of *A. dorsata* worker from *N. ceranae*-infected bee treated with 50% propolis extract (NC+PO) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

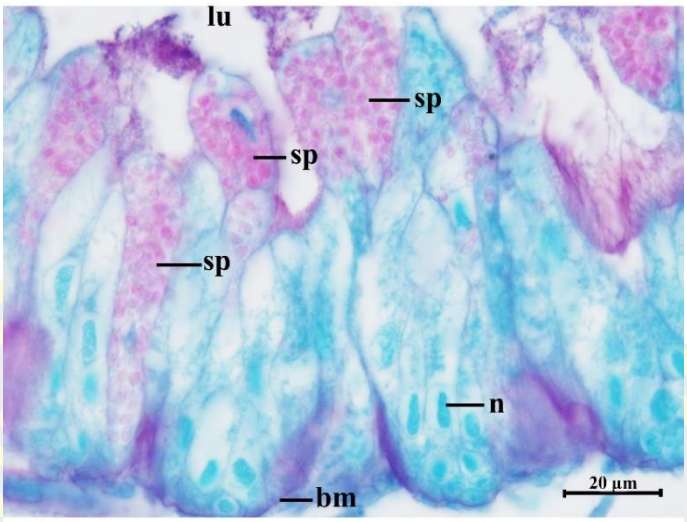

Figure 4.37 Light micrograph of infected midgut ventricular cells of *A. dorsata* worker from *N. ceranae*-infected bee treated with 0.5 ppm COS (NC+COS) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

For *A. mellifera*, the midgut ventricular cells of control bee not treated (CO, Figure 4.38), control bee treated with propolis (CO+PO, Figure 4.39), and control bee treated with COS (CO+COS, Figure 4.40) on day 14 p.i. showed no *N.ceranae* spore inside the ventricular cells of the honey bee, while *N. ceranae* spores were found in infected bee groups (NC, NC+PO, and NC+COS, Figures 4.41-4.43). Heavy infection was found in infected bee not treated (NC, Figure 4.41), and the spores were distributed throughout the cytoplasm of midgut ventricular cells. *Nosema* spores were found from the apical part to the bottom of the cell cytoplasm. The swollen cytoplasm could be seen. However, lower infection was found in infected bee treated with propolis (NC+PO, Figure 4.42) and infected bee treated with COS (NC+COS, Figure 4.43).

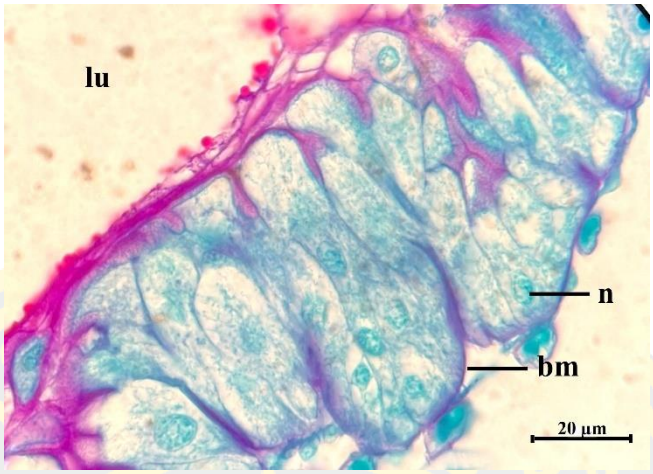

Figure 4.38 Light micrograph of midgut ventricular cells of *A. mellifera* worker from control bee not treated (CO) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

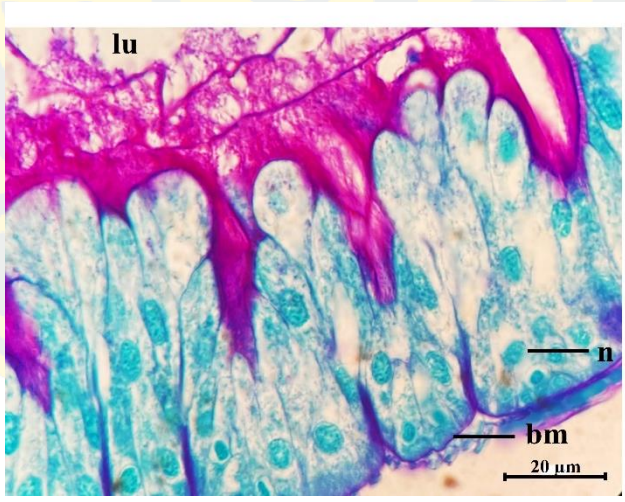

Figure 4.39 Light micrograph of midgut ventricular cells of *A. mellifera* worker from control bee treated with 50% propolis extract (CO+PO) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

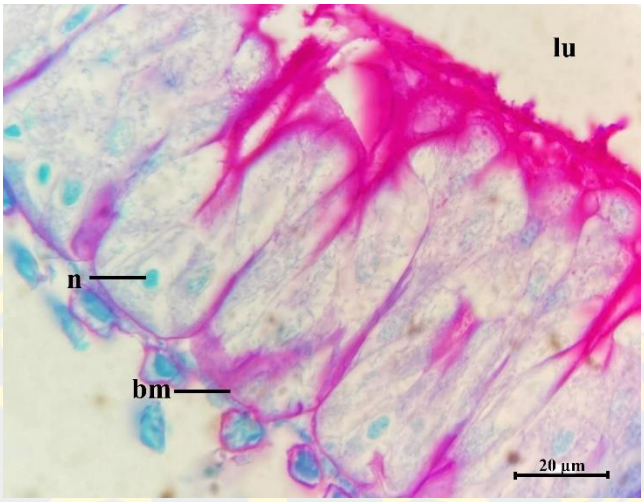

Figure 4.40 Light micrograph of midgut ventricular cells of *A. mellifera* worker from control bee treated with 0.5 ppm COS (CO+COS) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell.

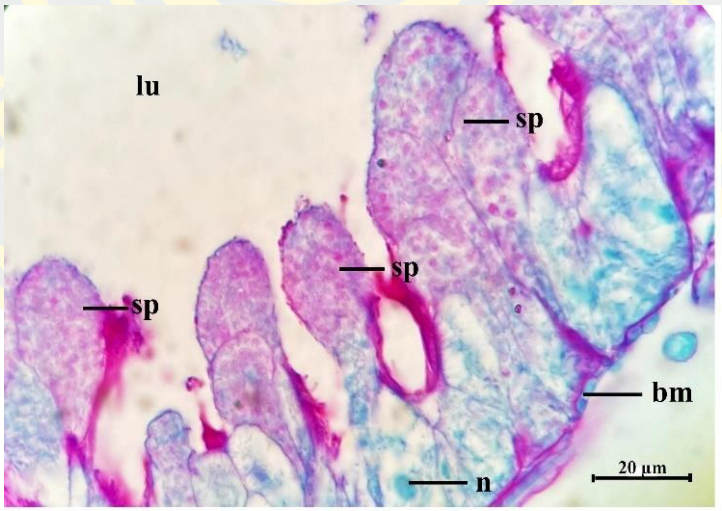

Figure 4.41 Light micrograph of infected midgut ventricular cells of *A. mellifera* worker from *N. ceranae*-infected bee not treated (NC) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

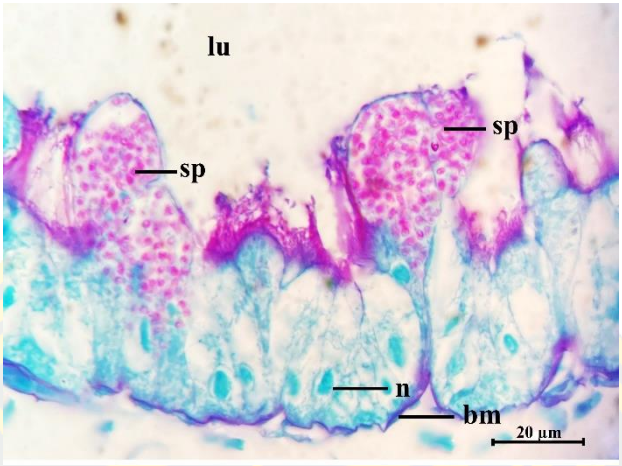

Figure 4.42 Light micrograph of infected midgut ventricular cells of *A. mellifera* worker from *N. ceranae*-infected bee treated with 50% propolis extract (NC+PO) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

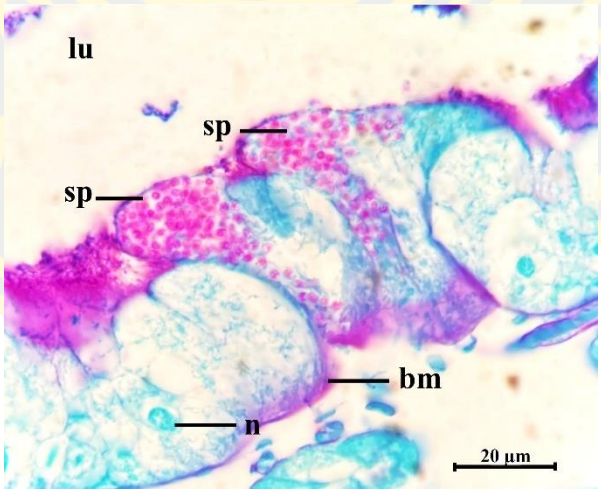

Figure 4.43 Light micrograph of infected midgut ventricular cells of *A. mellifera* worker from *N. ceranae*-infected bee treated with 0.5 ppm COS (NC+COS) on day 14 p.i. (PAS, 1,000×). Abbreviations: bm, basement membrane; lu, lumen of the midgut; n, nucleus of a ventricular cell; sp, *N. ceranae* spore.

4.3.5 Trehalose level in hemolymph

The trehalose levels in hemolymph of *A. dorsata* in CO, CO+PO, and CO+COS on day 3 post infection (p.i.) were 87.81 ± 2.03, 93.12 ± 5.02, and 92.14 ± 3.36 µg per bee, respectively, while NC, NC+PO, and NC+COS were 79.47 ± 2.27, 85.84 ± 3.27, and 84.47 ± 3.75 µg per bee, respectively which were not significant differences among the treatments ($F = 2.35$, $df = 11$, $p = 0.0105$, Figure 4.44). The trehalose levels in hemolymph of *A. mellifera* in CO, CO+PO, and CO+COS on day 3 p.i. were 89.59 ± 4.62, 95.04 ± 4.60, and 93.07 ± 4.82 µg per bee, respectively, while NC, NC+PO, and NC+COS were 68.60 ± 5.18, 83.57 ± 5.95, and 83.22 ± 4.55 µg per bee, respectively. The trehalose level of *A. mellfiera* in NC was significantly lower than *A. dorsata* in CO+PO and CO+COS ($F = 2.35$, $df = 11$, $p = 0.0105$).

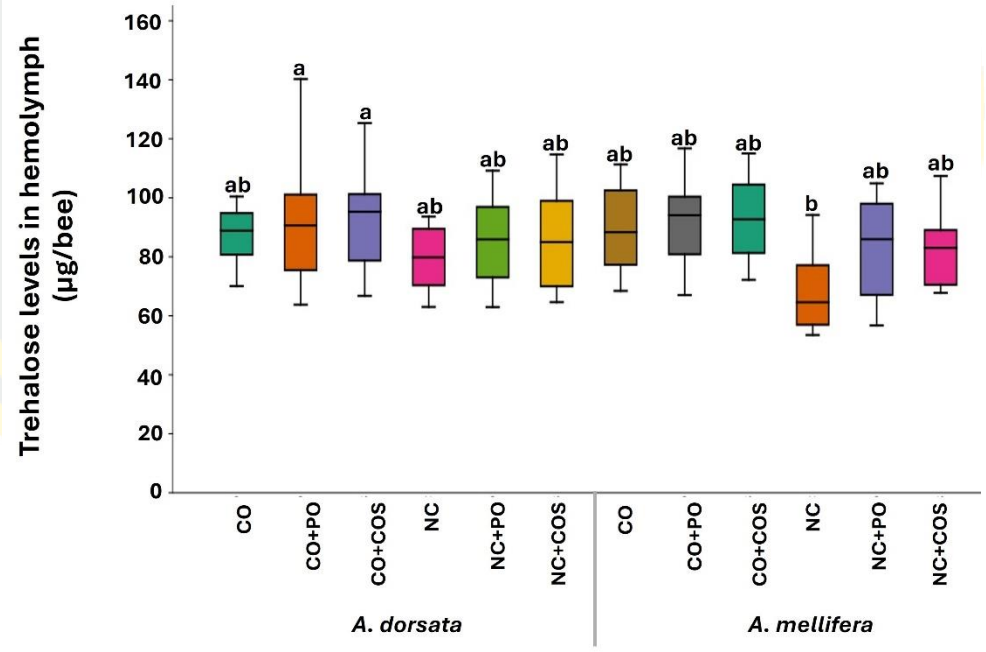

Figure 4.44 Box plots of trehalose levels in hemolymph (µg per bee) of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 3 p.i. The different letters above the vertical bar indicate significant differences among groups (One-way ANOVA; $F = 2.35$, $df = 11$, $p = 0.0105$).

On day 6 p.i. (Figure 4.45), the trehalose levels of *A. dorsata* in CO, CO+PO, CO+COS were 102.20 ± 3.47, 100.17 ± 2.96, and 97.21 ± 3.49 µg per bee, respectively, these results were significantly higher than trehalose levels of *A. dorsata* in NC, NC+PO, and NC+COS which were 66.58 ± 3.22, 83.20 ± 2.03, and 74.22 ± 2.56 µg per bee, respectively ($\chi^2 = 83.33$, $df = 11$, $p < 0.0001$). The trehalose levels of NC+PO was significantly higher than NC, but not significantly different from NC+COS. For *A. mellifera,* the trehalose levels of CO, CO+PO, CO+COS were 104.38 ± 5.73, 101.42 ± 2.79, and 102.03 ± 4.93 µg per bee, respectively, these results were significantly higher than trehalose levels of NC which was 60.54 ± 5.64 µg per bee. The trehalose levels of NC+COS (84.97 ± 3.90 µg per bee) was significantly higher than NC but not significantly different from NC+PO (81.43 ± 5.95 µg per bee).

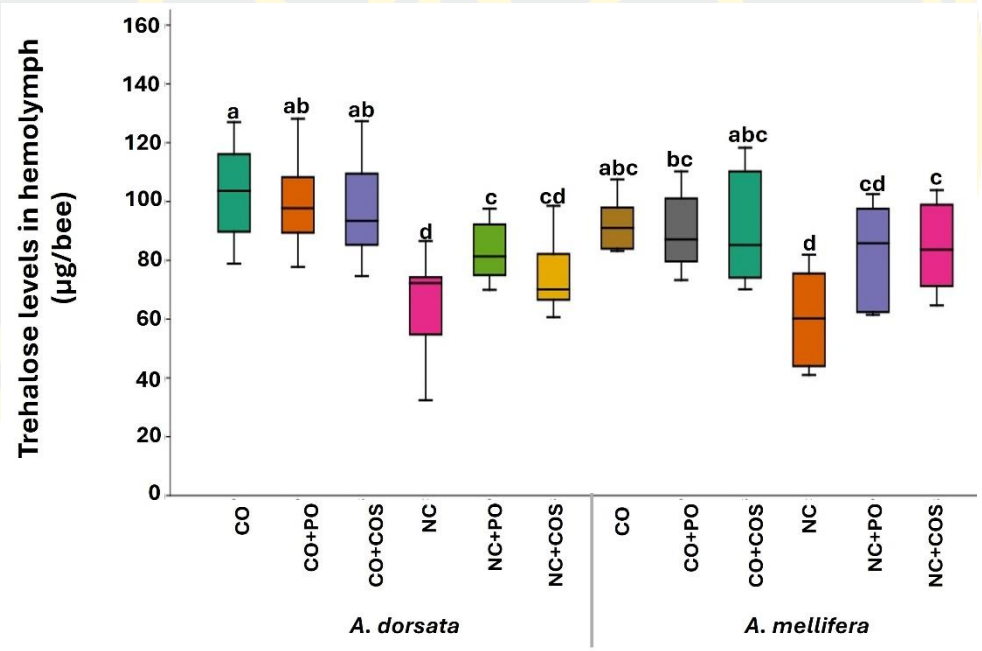

Figure 4.45 Box plots of trehalose levels in hemolymph (µg per bee) of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 6 p.i. The different letters above the vertical bar indicate significant differences among groups (Kruskal-Wallis test; $\chi^2 = 83.33$, $df = 11$, $p < 0.0001$).

On day 10 p.i. (Figure 4.46), the trehalose levels of *A. dorsata* in CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 111.18 ± 3.24, 106.95 ± 3.03, 113.36 ± 3.58, 63.19 ± 3.22, 87.59 ± 2.68, and 85.31 ± 3.71 µg per bee, respectively. In *A. mellifera*, the trehalose levels of CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 125.06 ± 4.39, 130.06 ± 7.15, 127.60 ± 5.09, 64.33 ± 3.76, 91.06 ± 4.25, and 92.34 ± 3.88 µg per bee, respectively. The trehalose levels of bees in NC+PO and NC+COS were significantly higher than those of NC in both honey bees species ($F = 31.68$, $df = 11$, $p < 0.0001$). However, the trehalose levels of NC+PO and NC+COS were significantly lower than those of control groups; CO, CO+PO, and CO+COS.

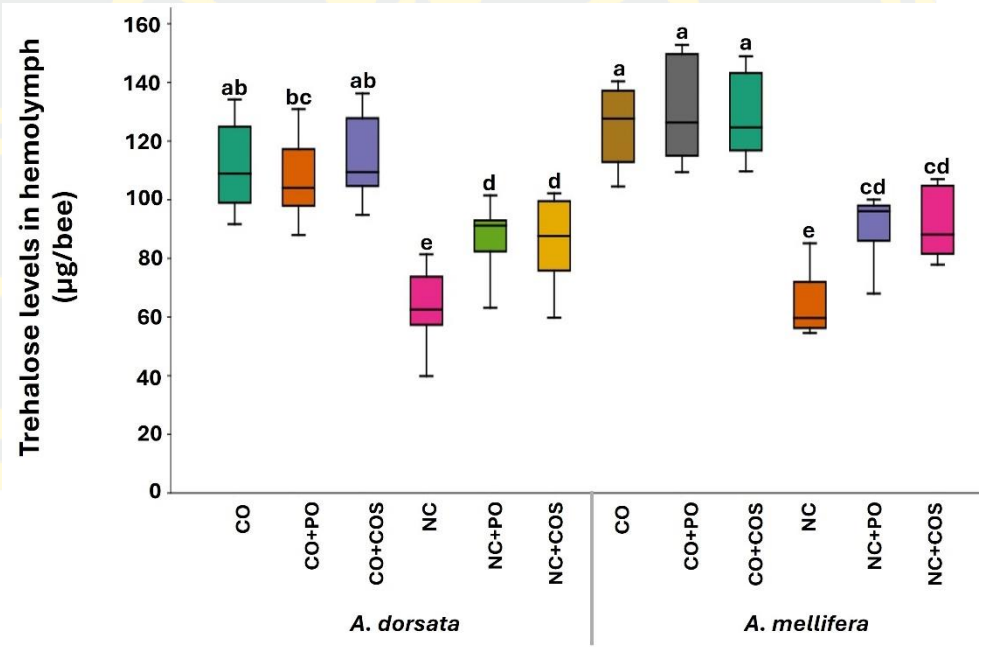

Figure 4.46 Box plots of trehalose levels in hemolymph (µg per bee) of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 10 p.i. The different letters above the vertical bar indicate significant differences among groups (One-way ANOVA; $F = 31.68$, $df = 11$, $p < 0.0001$).

On day 14 p.i. (Figure 4.47), the trehalose levels of *A. dorsata* in CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 110.14 ± 3.25, 103.88 ± 3.05, 106.53 ± 2.63, 59.53 ± 3.54, 79.56 ± 3.04, and 80.23 ± 3.52 µg per bee, respectively. For *A. mellifera*, the trehalose levels of CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 106.76 ± 7.15, 107.65 ± 6.94, 101.77 ± 3.77, 55.15 ± 4.57, 82.17 ± 4.65, and 85.30 ± 3.78 µg per bee, respectively.

There was no significant difference among *A. dorsata* and *A. mellifera* in CO, CO+PO, and CO+COS. The trehalose levels of *A. dorsata* and *A. mellifera* in NC+PO and NC+COS were significantly higher than NC ($\chi^2 = 106.80$, $df = 11$, $p < 0.0001$), but significantly lower than those of CO, CO+PO, and CO+COS.

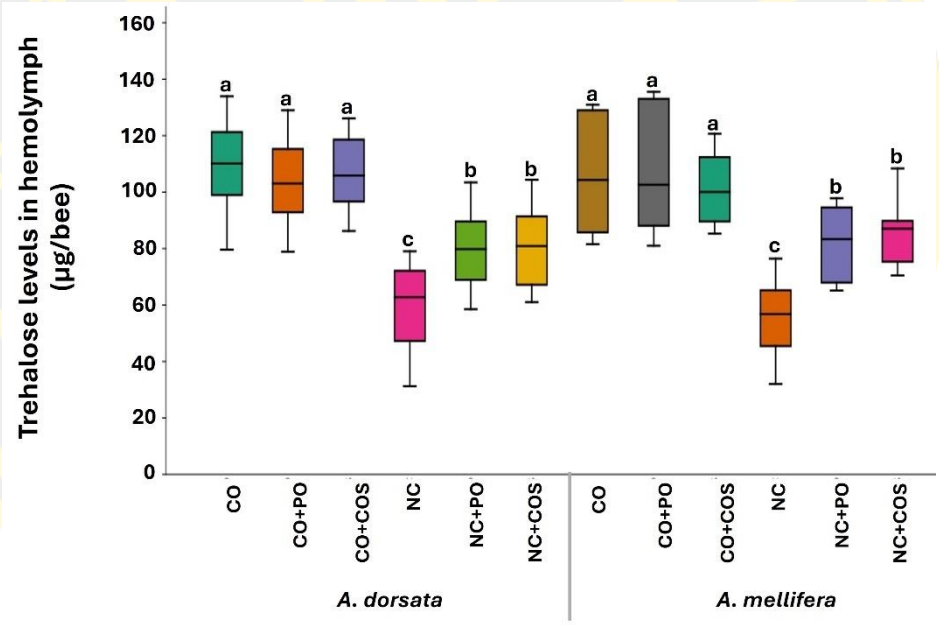

Figure 4.47 Box plots of trehalose levels in hemolymph (µg per bee) of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 14 p.i. The different letters above the vertical bar indicate significant differences among groups (Kruskal-Wallis test; $\chi^2 = 106.80$, $df = 11$, $p < 0.0001$).

4.3.6 Hypopharyngeal gland protein contents

The protein content of hypopharyngeal gland at day 3 p.i. of *A. dorsata* in CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 1,062.68 ± 49.92, 1,015.91 ± 47.94, 1,068.83 ± 55.87, 813.15 ± 46.19, 882.16 ± 44.48, and 913.63 ± 43.55 µg per bee, respectively (Figure 4.48). The protein content of bees in NC was significantly lower than bees in CO and CO+COS ($F = 2.95$, $df = 11$, $p = 0.0014$). In *A. mellifera,* the hypopharyngeal gland protein content of CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 988.89 ± 45.85, 986.64 ± 55.72, 935.24 ± 70.13, 797.48 ± 48.50, 912.63 ± 63.91, and 917.77 ± 66.55 µg per bee, respectively.

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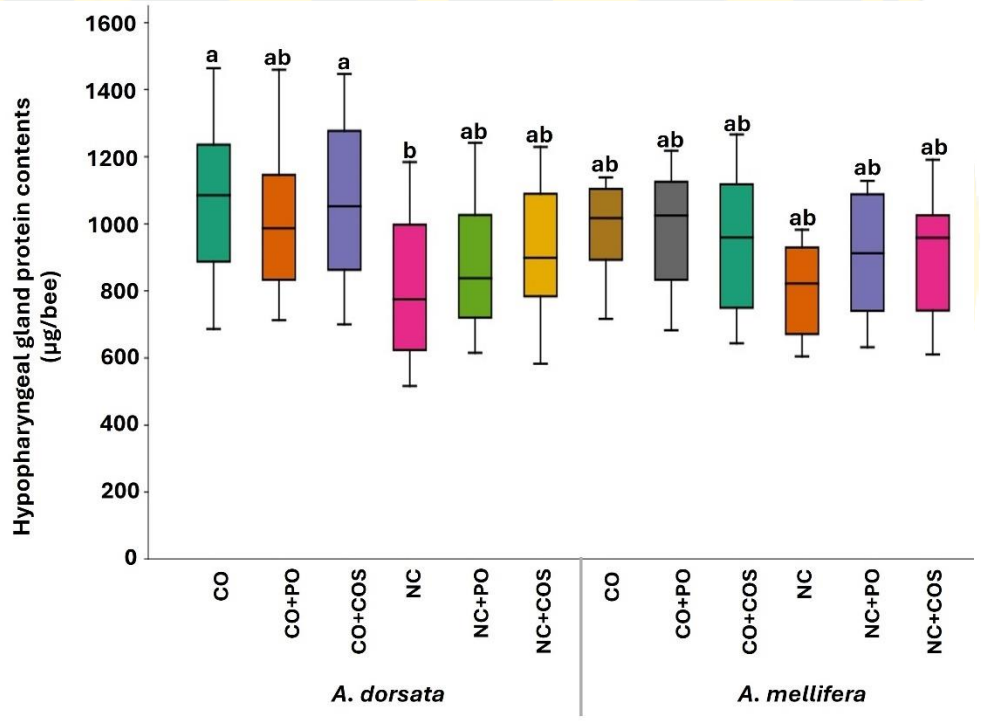

Figure 4.48 Box plots of hypopharyngeal gland protein contents (µg per bee) of *A. dorsata* and *A. mellifera* workers; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 3 p.i. The different letters above the vertical bar indicate significant differences among groups (One-way ANOVA; $F = 2.95$, $df = 11$, $p = 0.0014$).

On day 6 p.i. (Figure 4.49), the protein contents of *A. dorsata* in CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 807.10 ± 30.99, 767.68 ± 34.19, 774.69 ± 30.74, 511.85 ± 20.37, 647.80 ± 22.80, and 639.74 ± 28.67 µg per bee, respectively. For *A. mellifera,* the hypopharyngeal gland protein content of CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 862.47 ± 57.57, 865.16 ± 70.14, 886.10 ± 92.31, 508.32 ± 28.55, 597.04 ± 41.02, and 632.04 ± 58.83 µg per bee, respectively.

There was no significant difference in the protein content among *A. dorsata* and *A. mellifera* in CO, CO+PO, and CO+COS ($\chi^2 = 72.89$, $df = 11$, $p < 0.0001$). The protein contents of *A. dorsata* in NC+PO and NC+COS were significantly higher than NC. However, there was no significant difference among *A. mellifera* in NC, NC+PO, and NC+COS.

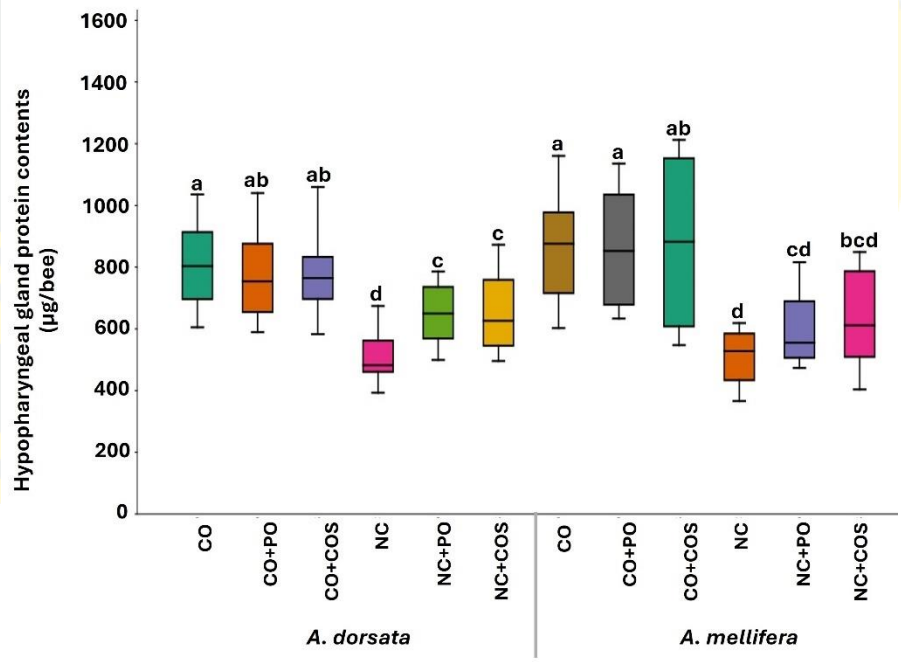

Figure 4.49 Box plots of hypopharyngeal gland protein contents (µg per bee) of *A. dorsata* and *A. mellifera* workers; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 6 p.i. The different letters above the vertical bar indicate significant differences among groups (Kruskal-Wallis test; $\chi^2 = 72.89$, $df = 11$, $p < 0.0001$).

On day 10 p.i. (Figure 4.50), the protein contents of *A. dorsata* in CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 762.42 ± 30.99, 737.24 ± 22.94, 725.64 ± 27.75, 413.47 ± 18.28, 580.52 ± 23.36, and 602.86 ± 23.99 µg per bee, respectively. For *A. mellifera*, the protein contents of CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 788.58 ± 56.35, 752.25 ± 59.90, 744.43 ± 57.78, 387.37 ± 29.69, 553.16 ± 23.94, and 551.35 ± 33.60 µg per bee, respectively.

There was no significant difference in the protein content among *A. dorsata* and *A. mellifera* in CO, CO+PO, and CO+COS ($\chi^2 = 86.05$, $df = 11$, $p < 0.0001$). The protein contents of bees in NC+PO and NC+COS were significantly higher than NC for both bee species. However, the protein content of NC+PO and NC+COS were significantly lower than those of CO, CO+PO, and CO+COS.

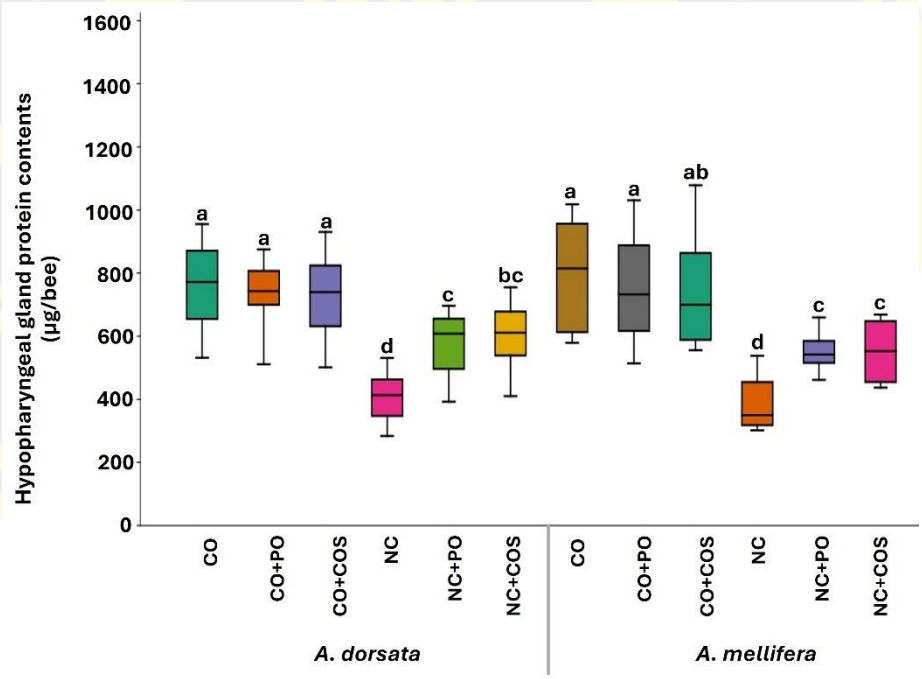

Figure 4.50 Box plots of hypopharyngeal gland protein contents (µg per bee) of *A. dorsata* and *A. mellifera* workers; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 10 p.i. The different letters above the vertical bar indicate significant differences among groups (Kruskal-Wallis test; $\chi^2 = 86.05$, $df = 11$, $p < 0.0001$).

On day 14 p.i. (Figure 4.51), the protein contents of *A. dorsata* in CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 727.51 ± 24.70, 664.63 ± 26.93, 669.82 ± 23.53, 362.08 ± 15.68, 510.82 ± 17.92, and 508.18 ± 16.06 µg per bee, respectively. For *A. mellifera*, the protein contents of CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 754.60 ± 60.87, 749.93 ± 65.66, 768.17 ± 73.84, 313.27 ± 30.82, 515.26 ± 32.24, and 511.73 ± 35.59 µg per bee, respectively.

The protein content of *A. dorsata* and *A. mellifera* in NC+PO and NC+COS were significantly higher than NC ($\chi^2 = 100.80$, $df = 11$, $p < 0.0001$), but significantly lower than those of CO, CO+PO, and CO+COS.

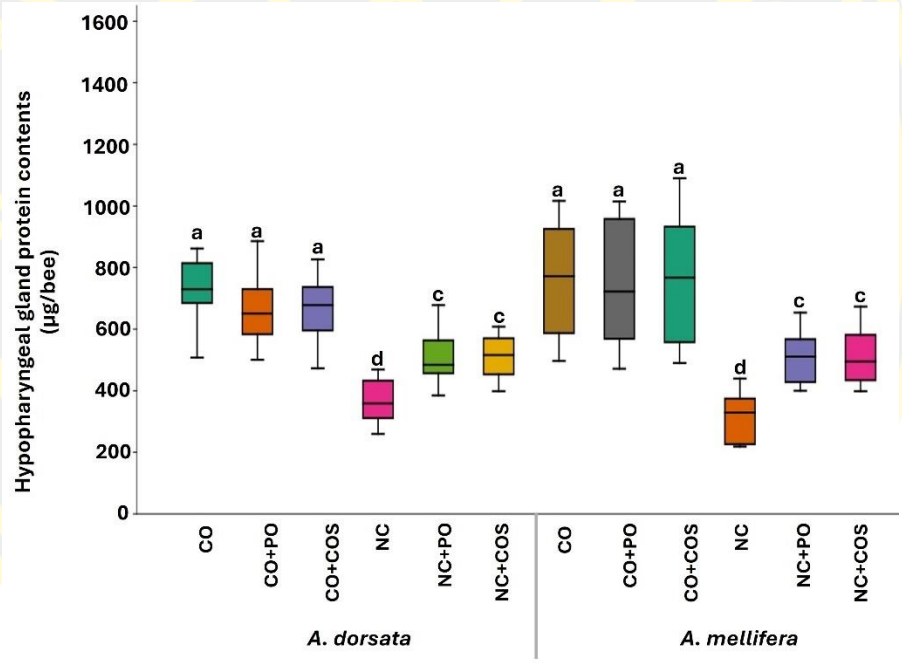

Figure 4.51 Box plots of hypopharyngeal gland protein contents (µg per bee) of *A. dorsata* and *A. mellifera* workers; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 14 p.i. The different letters above the vertical bar indicate significant differences among groups (Kruskal-Wallis test; $\chi^2 = 100.80$, $df = 11$, $p < 0.0001$).

4.3.7 Midgut proteolytic enzyme activities

The total of midgut proteolytic enzyme activities of *A. dorsata* in CO, CO+PO, and CO+COS on day 3 p.i. were 0.4080 ± 0.0084, 0.4082 ± 0.0066, and 0.4053 ± 0.0085 OD_{440}, respectively, whereas NC, NC+PO, and NC+COS were 0.3565 ± 0.0161, 0.3938 ± 0.0083, and 0.3897 ± 0.0070 OD_{440}, respectively (Figure 4.52). The proteolytic enzyme activities of *A. dorsata* in NC was significantly difference from *A. dorsata* in CO, CO+PO, and CO+COS, but not different from NC+PO and NC+COS ($\chi^2 = 23.18$, $df = 11$, $p = 0.0167$).

For *A. mellifera*, the total of midgut proteolytic enzyme activities of CO, CO+PO, and CO+COS were 0.4587 ± 0.0283, 0.4582 ± 0.0362, and 0.4253 ± 0.0287 OD_{440}, respectively, while NC, NC+PO, and NC+COS were 0.3706 ± 0.0204, 0.3670 ± 0.0173, and 0.3742 ± 0.0219 OD_{440}, respectively. There was no significant difference in proteolytic enzyme activity among *A. mellifera* in NC, CO, CO+PO and CO+COS.

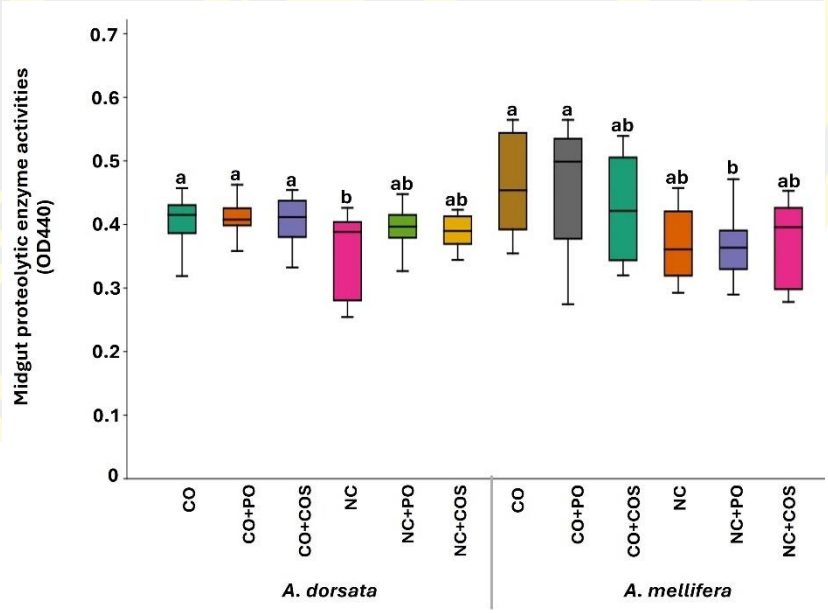

Figure 4.52 Box plots of total midgut proteolytic enzyme activities (OD_{440}) of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 3 p.i. The different letters above the vertical bar indicate significant differences among groups (Kruskal-Wallis test; $\chi^2 = 23.18$, $df = 11$, $p = 0.0167$).

On day 6 p.i. (Figure 4.53), the total midgut proteolytic enzyme activities of *A. dorsata* in CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 0.3731 ± 0.0110, 0.3355 ± 0.0111, 0.3334 ± 0.0110, 0.2545 ± 0.0150, 0.3047 ± 0.0136, and 0.2853 ± 0.0145 OD_{440}, respectively. For *A. mellifera*, the total midgut proteolytic enzyme activities of CO, CO+PO, CO+COS, NC, NC+PO, and NC+COS were 0.3253 ± 0.0242, 0.3094 ± 0.00290, 0.3257 ± 0.0206, 0.2062 ± 0.0108, 0.2588 ± 0.0229, and 0.2767 ± 0.0221 OD_{440}, respectively.

The total midgut proteolytic enzyme activities of *A. dorsata* and *A. mellifera* in CO, CO+PO, and CO+COS were significantly higher than NC ($F = 7.93$, $df = 11$, $p < 0.0001$). Moreover, the total midgut proteolytic enzyme activities of *A. dorsata* in NC+PO and NC+COS were not significant difference from *A. dorsata* in NC. The same trend was found in A. mellifera, the total midgut proteolytic enzyme activities of *A. mellifera* in NC+PO and NC+COS were not significant difference from *A. mellifera* in NC.

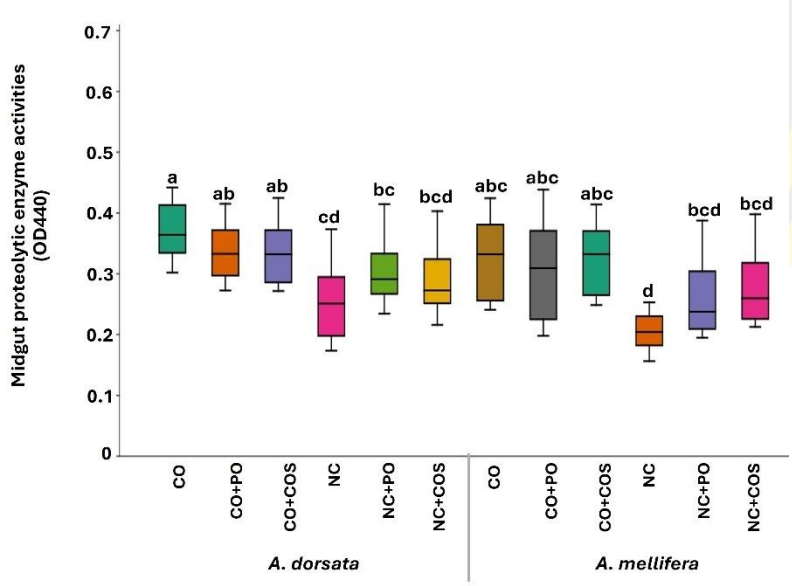

Figure 4.53 Box plots of total midgut proteolytic enzyme activities (OD_{440}) of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 6 p.i. The different letters above the vertical bar indicate significant differences among groups (One-way ANOVA; $F = 7.93$, $df = 11$, $p < 0.0001$).

On day 10 p.i. (Figure 4.54), There were no significant difference in the midgut proteolytic enzyme activity among *A. dorsata* and *A. mellifera* in CO, CO+PO, and CO+COS ($F = 28.28$ $df = 11$, $p < 0.0001$). The proteolytic enzyme activities of *A. dorsata* in NC+PO and NC+COS (0.1709 ± 0.0093 and 0.1818 ± 0.0094 OD_{440}, respectively) were significantly higher than *A. dorsata* in NC (0.1123 ± 0.0088 OD_{440}) ($F = 28.28$ $df = 11$, $p < 0.0001$). However, proteolytic enzyme activities of *A. dorsata* in NC+PO and NC+COS were significantly lower than those of *A. dorsata* in CO, CO+PO, and CO+COS which were 0.2614 ± 0.0087, 0.2511 ± 0.0089, and 0.2550 ± 0.0105 OD_{440}, respectively.

For *A. mellifera,* the proteolytic enzyme activities of NC+PO and NC+COS (0.1903 ± 0.0204 and 0.1696 ± 0.0157 OD_{440}, respectively) were significantly higher than NC (0.0839 ± 0.0072 OD_{440}) ($F = 19.27$, $df = 5$, $p < 0.0001$). However, proteolytic enzyme activities of NC+PO and NC+COS were significantly lower than those of CO, CO+PO, and CO+COS which were 0.2789 ± 0.0219, 0.2748 ± 0.0213 and 0.2730 ± 0.0165 OD_{440}, respectively.

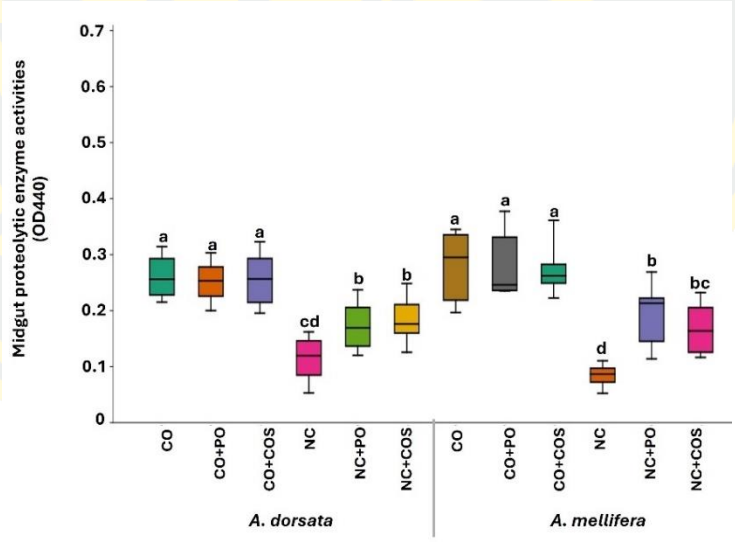

Figure 4.54 Box plots of total midgut proteolytic enzyme activities (OD_{440}) of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 10 p.i. The different letters above the vertical bar indicate significant differences among groups (One-way ANOVA; $F = 28.28$, $df = 11$, $p < 0.0001$).

On day 14 p.i. (Figure 4.55), There were no significant difference in the midgut proteolytic enzyme activity among *A. dorsata* and *A. mellifera* in CO, CO+PO, and CO+COS ($F = 31.17$, $df = 11$, $p < 0.0001$). The proteolytic enzyme activities of *A. dorsata* in NC+PO and NC+COS (0.1452 ± 0.0092 and 0.1464 ± 0.0081 OD_{440}, respectively) were significantly higher than *A. dorsata* in NC (0.0766 ± 0.0053 OD_{440}) ($F = 31.17$, $df = 11$, $p < 0.0001$). However, proteolytic enzyme activities of *A. dorsata* in NC+PO and NC+COS were significantly lower than those of *A. dorsata* in CO, CO+PO, and CO+COS which were 0.2225 ± 0.0084, 0.2144 ± 0.0088 and 0.2258 ± 0.0086 OD_{440}, respectively.

For *A. mellifera,* the proteolytic enzyme activities of NC+PO and NC+COS (0.1245 ± 0.0099 and 0.1254 ± 0.0132 OD_{440}, respectively) were significantly higher than NC (0.0609 ± 0.0088 OD_{440}) ($F = 19.27$, $df = 5$, $p < 0.0001$). However, proteolytic enzyme activities of NC+PO and NC+COS were significantly lower than those of CO, CO+PO, and CO+COS which were 0.1950 ± 0.0156, 0.1927 ± 0.0166 and 0.1917 ± 0.0205 OD_{440}, respectively.

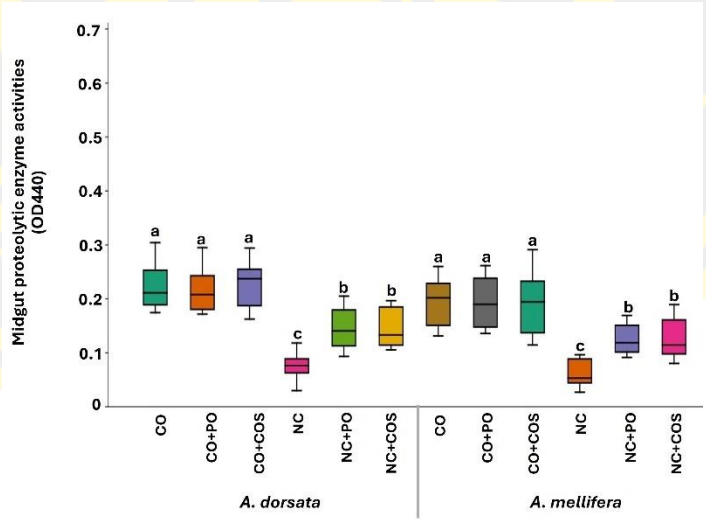

Figure 4.55 Box plots of total midgut proteolytic enzyme activities (OD_{440}) of *A. dorsata* and *A. mellifera*; control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on day 14 p.i. The different letters above the vertical bar indicate significant differences among groups (One-way ANOVA; $F = 31.17$, $df = 11$, $p < 0.0001$).

4.5 Experiment IV: To measure the expression of immune-related genes of *A. dorsata* and *A. mellifera* workers response to the infection of *N. ceranae* and after treating with propolis and COS.

4.5.1 The expression of immune-related genes of *A. dorsata* and *A. mellifera* workers

The expression levels of the antimicrobial peptide-encoding genes, apidaecin, defensin-1 and hymenoptaecin of *A. dorsata* and *A. mellifera* were measured on days 3, 6, 10 and 14 p.i. The overall expression levels of apidaecin and defensin-1 (Figure 4.56 and 4.57) of both honey bee species were not influence by *N. ceranae* infection when compared to control bees (CO). The significant down-regulation in hymenoptaecin was observed in *A. dorsata* after dosed with *N. ceranae* 6 days (X^2 = 11.32, df = 5, p = 0.0454, Figure 4.58a). However, the up-regulation in hymenoptaecin was found in *A. mellifera* after dosed with *N. ceranae* 14 days (X^2 = 15.68, df = 5, p = 0.0078, Figure 4.58b).

The expression levels of apidaecin and hymenoptaecin (Figure 4.56 and 4.58) were up-regulated in infected bees treated with propolis extract for both honey bee species. However, the significant up-regulation was found on day 6 p.i. for *A. dorsata* (X^2 = 11.87, df = 5, p = 0.0366) and day 3 p.i. for *A. mellifera* (X^2 = 13.65, df = 5, p = 0.0180) when compared to infected bees not treated. Moreover, the expression of hymenoptaecin (Figure 4.58b) of *A. mellifera* dosed with *N. ceranae* and treated with COS was significant up-regulated on day 3 p.i. compared to untreated bees (X^2 = 17.84, df = 5, p = 0.0031).

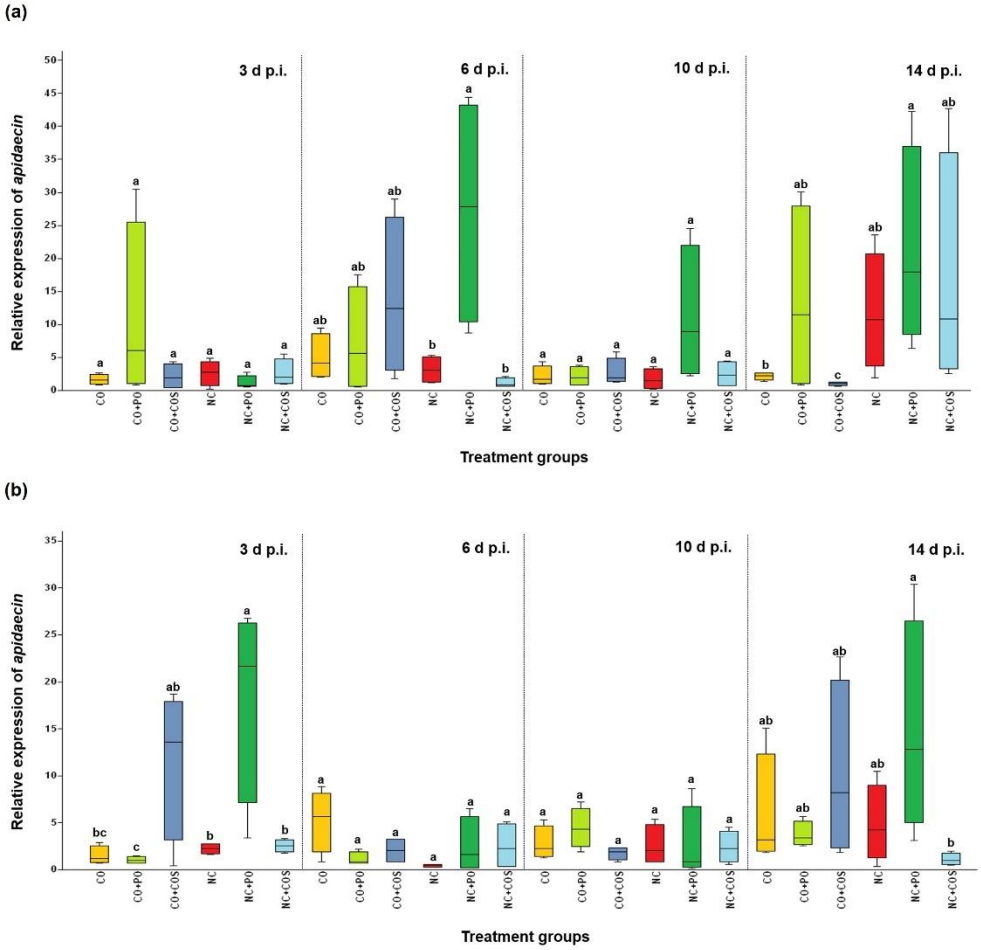

Figure 4.56 Box plots of relative expression of apidaecin of *A. dorsata* (a) and *A. mellifera* (b) workers from control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on days 3, 6, 10, and 14 p.i. The different letters above vertical bars indicate significantly different from one another (Kruskal-Wallis test apidaecin; $\chi^2 = 3.31$, $df = 5$, $p = 0.6519$, $\chi^2 = 11.87$, $df = 5$, $p = 0.0366$, $\chi^2 = 5.00$, $df = 5$, $p = 0.4159$, and $\chi^2 = 12.12$, $df = 5$, $p = 0.0332$ for *A. dorsata* on days 3, 6, 10, and 14 p.i., respectively, $\chi^2 = 13.65$, $df = 5$, $p = 0.0180$, $\chi^2 = 8.56$, $df = 5$, $p = 0.1277$, $\chi^2 = 3.958$, $df = 5$, $p = 0.5553$, and $\chi^2 = 9.53$, $df = 5$, $p = 0.0397$ for *A. mellifera* on days 3, 6, 10, and 14 p.i., respectively).

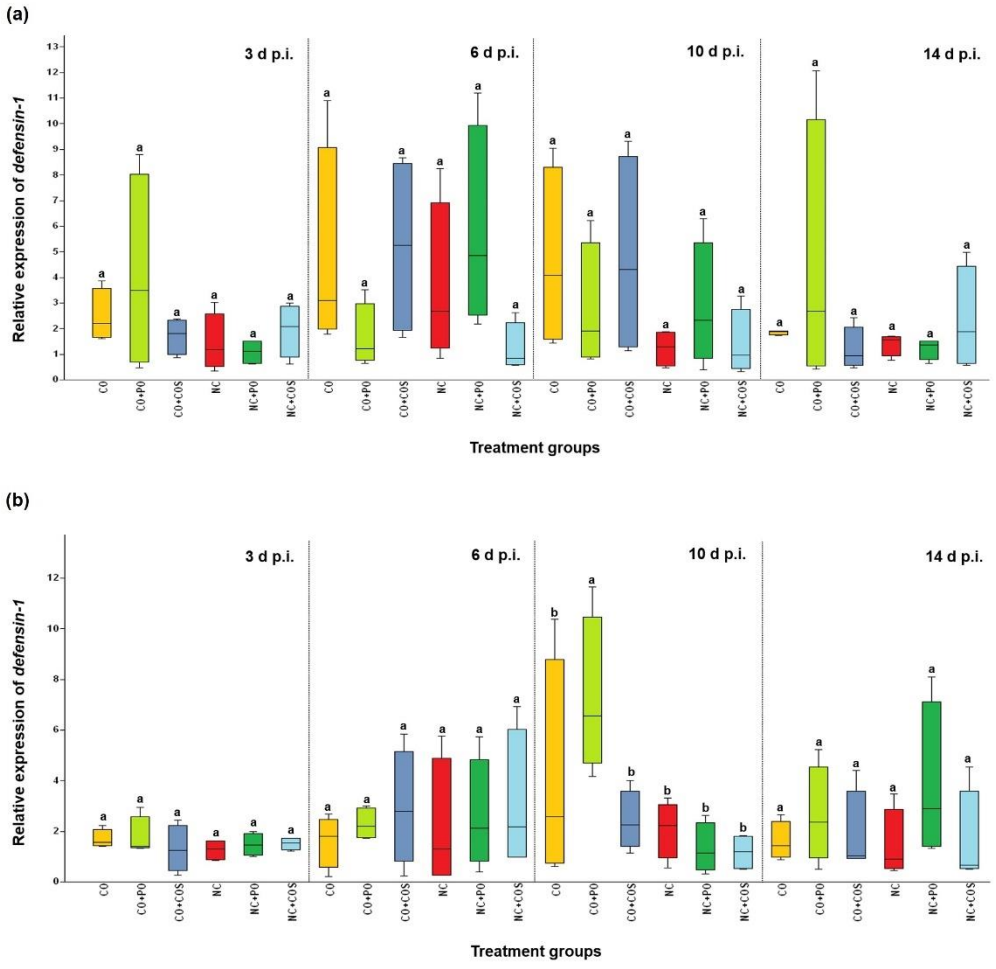

Figure 4.57 Box plots of relative expression of defensin-1 of *A. dorsata* (a) and *A. mellifera* (b) workers from control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on days 3, 6, 10, and 14 p.i. The different letters above vertical bars indicate significantly different from one another (Kruskal-Wallis test apidaecin; $\chi^2 = 4.96$, $df = 5$, $p = 0.4208$, $\chi^2 = 9.67$, $df = 5$, $p = 0.0848$, $\chi^2 = 5.55$, $df = 5$, $p = 0.3525$, and $\chi^2 = 3.813$, $df = 5$, $p = 0.5765$ for *A. dorsata* on days 3, 6, 10, and 14 p.i., respectively, $\chi^2 = 1.718$, $df = 5$, $p = 0.8865$, $\chi^2 = 1.712$, $df = 5$, $p = 0.8871$, $\chi^2 = 11.18$, $df = 5$, $p = 0.0478$, and $\chi^2 = 5.01$, $df = 5$, $p = 0.4147$ for *A. mellifera* on days 3, 6, 10, and 14 p.i., respectively).

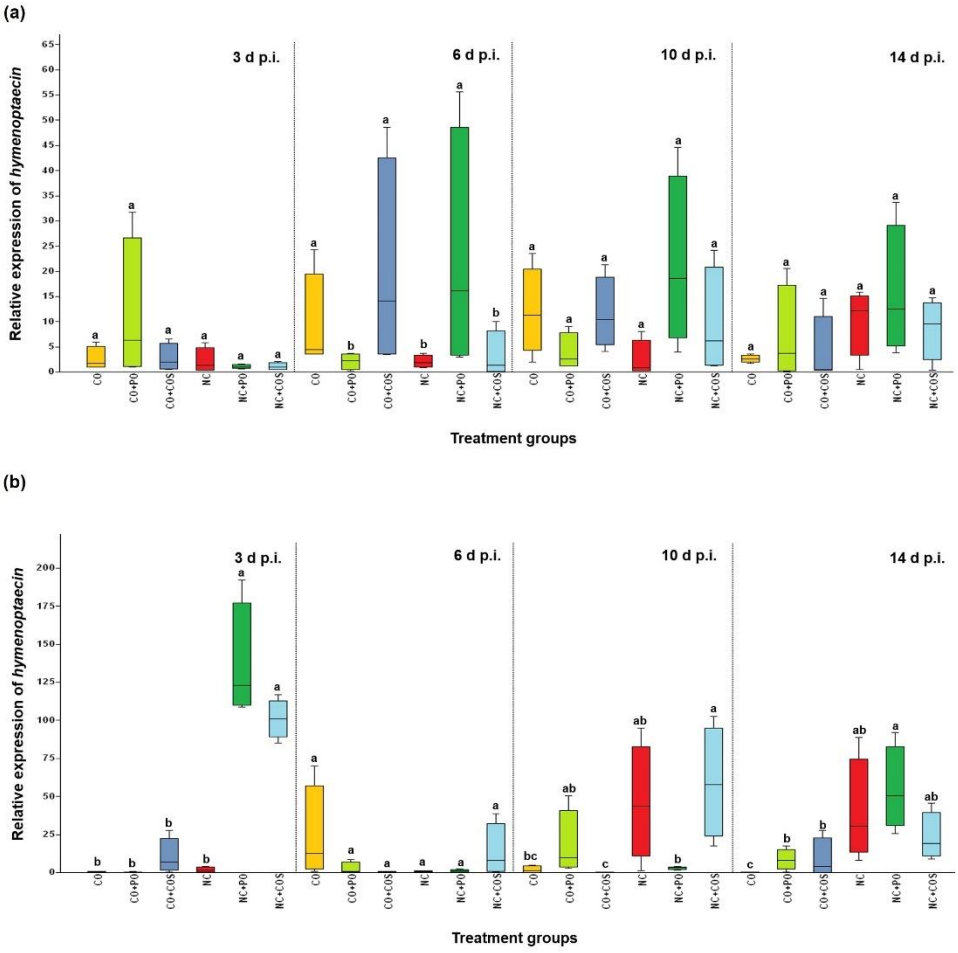

Figure 4.58 Box plots of relative expression of hymenoptaecin of *A. dorsata* (a) and *A. mellifera* (b) workers from control bees not treated (CO), control bees treated with propolis (CO+PO), control bees treated with COS (CO+COS), *N. ceranae*-infected bees not treated (NC), infected bees treated with propolis (NC+PO), and infected bees treated with COS (NC+COS) on days 3, 6, 10, and 14 p.i. The different letters above vertical bars indicate significantly different from one another (Kruskal-Wallis test apidaecin; χ^2 = 4.01, df = 5, p = 0.5480, χ^2 = 11.32, df = 5, p = 0.0454, χ^2 = 10.84, df = 5, p = 0.0547, and χ^2 = 6.32, df = 5, p = 0.2763 for *A. dorsata* on days 3, 6, 10, and 14 p.i., respectively, χ^2 = 17.84, df = 5, p = 0.0031, χ^2 = 7.14, df = 5, p = 0.2100, χ^2 = 15.73, df = 5, p = 0.0077, and χ^2 = 15.68, df = 5, p = 0.0078 for *A. mellifera* on days 3, 6, 10, and 14 p.i., respectively).

CHAPTER 5
DISCUSSION AND CONCLUSION

Nosema ceranae is a microsporidian pathogen that was first discovered in *Apis cerana*. This parasite recently can be found to infect all honey bee species. There was a report that *N. ceranae* isolated from different hosts have differences in their ability to infect honey bees (Chaimanee & Chantawannakul, 2016), and might cause differences in virulence on *A. dorsata* workers.

Spores of *N. ceranae* isolated from *A. cerana* had a higher ID_{50} (550,000 spores) than *N. ceranae* isolated from *A. florea* (54,000 spores), *A. dorsata,* and *A. mellifera* (20,000 spores). The ID_{50} in this study was higher than previous study, which required 85 *N. ceranae* spores per bee to infect *A. mellifera* (Forsgren and Fries, 2010). Another study found that ID_{50} was approximately 10,053 spores and ID_{100} was approximately 506,778 spores for *N. ceranae* infected in *A. mellifera* (Huang, Solter, Aronstein, & Huang, 2015). A dose of 5×10^5 spores caused 100% infection in *A. dorsata* workers for *N. ceranae* isolated from *A. dorsata*, *A. florea*, and *A. mellifera*. However, *N. ceranae* spores isolated from *A. cerana* can cause 100% infection in *A. dorsata* workers when dosed with 1×10^6 spores per bee. The workers of *A. dorsata* inoculated with *N. ceranae* isolated from *A. cerana* had lower infectivity compared to other *N. ceranae* isolates when given spores at concentration of 1×10^4 and 1×10^5 spores per bee. These findings indicated that *N. ceranae* isolated from *A. cerana* might have a lower ability to infect *A. dorsata* workers. Previous research showed that *N. ceranae* isolated from *A. mellifera* was higher infective than isolated from *A. cerana* after inoculation in both *A. mellifera* and *A. cerana* workers (Chaimanee & Chantawannakul, 2016). However, a study found no difference in genetic relationship between *N. ceranae* isolated from *A. cerana* and *A. mellifera*. Therefore, the virulence of these two isolates should be similar (Chaimanee et al., 2010). However, the different in infection ability might be due to the various factors such as host susceptibility, defense mechanisms at the individual level, and natural co-evolution between host and parasites, which can influence the ability of infection (Chaimanee et al., 2013; Dussaubat et al., 2013; Van der Zee et al., 2014). The

lowered infection of *N. ceranae* isolated from *A. cerana* in this investigation could be attributed to the differences in genetic variations which is related to honey bee host species. Peters, Suwannapong, Pelin, and Corradi (2019) reported that Thai *N. ceranae* isolated from *A. cerana* showed lower loss of heterozygosity (LOH) region compared to *N. ceranae* isolated from *A. mellifera*. The LOH regions affect a pathogen's ability to adapt to new environment. This could explain why *N. ceranae* isolated from *A. cerana* is less ability to infect *A. dorsata.*

All *Nosema* isolates enhanced mortality of *A. dorsata* workers. This result corresponds with previous studies in *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* (Sanchai Naree et al., 2021; Ponkit et al., 2021; Suwannapong, Yemor, et al., 2011) which indicated that *N. ceranae*-infected bees had lower survival than healthy bees or control bees in laboratory condition. In this study, the significant difference in mortality was found in bees infected by *N. ceranae* isolated from *A. mellifera* at 5×10^5 spores per bee. Here, *N. ceranae* isolated from *A. mellifera* under the dose of ID_{100} (5×10^5 spores per bee) was used in the next experiment to ensure that all bees will get infection.

The workers of *A. dorsata* infected by *N. ceranae* isolated from *A. mellifera* had low proteolytic enzyme activity in their midgut. These might because of cell damaging caused by *N. ceranae* infection. The proteolytic enzyme activity of infected bees was decreased with time of infection. The lower protein content of hypopharyngeal gland also found in infected bees, and trend to decrease when with time of infection. Furthermore, the trehalose levels in hemolymph of *N. ceranae*-infected bees were lower than healthy bees.

Protein digestion impairment might contribute to the lack of hypopharyngeal gland development in infected bees. One study also showed that *N. ceranae*-infected bees had the reduction in hypopharyngeal gland sizes (Naree et al., 2021c). As a result, there is a paucity of nutrients for protein synthesis in the hypopharyngeal glands. This gland is essential for the production of royal jelly which is fed to honey bee larvae and queen. The problem with these glands will result in a lack of food quality and a weak colony (Ahmad et al., 2020; Kim et al., 2022). This study also measured the protein content of hypopharyngeal gland and found that *N. ceranae*-infected bee groups had lower protein content than control bees. The findings agree

with previous research which demonstrated that infected bees had a lower protein content in their hypopharyngeal glands (Naree et al., 2021c; Ponkit et al., 2021; Suwannapong et al., 2018).

Honey bees need mainly carbohydrates to provide energy for foraging and other activities. Carbohydrates such as glucose is a major fuel used by honey bees, and trehalose is a major carbohydrate storage molecule in bee hemolymph (Zoltowska et al., 2005). It is stored in the fat body and released into the bee haemolymph to be metabolized into glucose which then enters to the Krebs cycle and produces ATP (Aliferis et al., 2012). When trehalose in bee hemolymph was drop, the nectar from honey bee crop will passed through the midgut. The sucrose in nectar was metabolized into fructose and glucose and transported to the hemolymph. Then glucose was converted to trehalose in fat body (Reyes-DelaTorre, Teresa, & Rafael, 2012). In infected bees, *Nosema* spores directly draw energy and nutrient from host for their proliferation, and damage the ventricular cells of the host (Goblirsch, 2017; Suwannapong et al., 2010; Suwannapong, Yemor, et al., 2011). Therefore, the production of digestive enzymes and the food absorption of honey bee is impaired (Jack et al., 2016; Ponkit et al., 2021) resulting in the reduction of the substrates for various biosynthesis for honey bee. This situation will be force the honey bee to confront energetic stress.

The *Nosema* infection occurs mostly through ingestion of spores when honey bees are eating contaminated food or water and when they are cleaning up fecal material from infected bees. *N. ceranae* spores have been found in the pollen loads of bees, which include some regurgitated nectar from the crop and could be regurgitated to other colony members during food exchange, providing a mechanism for oral-oral transmission (Chen et al., 2008; Higes et al., 2008; Smith, 2012). The spores geminate within the midgut and release polar tubes that transfer their sporoplasm into midgut epithelial cells where they generate more spores. The spores can be passed to the environment through sputum and feces, providing new sources of the infection through cleaning and feeding activities in the colonies (Chen et al., 2008; Chen et al., 2009).

N. ceranae can infect both *A. mellifera* and *A. dorsata* workers and develop in these bee species since the infectivity and infection rate of infected bees were high.

It seems that *N. ceranae* develop very well when infect *A. mellifera* since the spore load of *N. ceranae* in this bee species was higher than *A. dorsata.* Our result was corresponded with previous research that reported the spore loads of *N. ceranae* infected *A. mellifera* was higher than the Asian honey bee species, *A. cerana* (Sinpoo et al., 2018). Previous studies indicated that infection by *N. ceranae* cause lower levels in trehalose sugar in bee haemolymph, hypopharyngeal gland protein contents, and proteolytic enzyme activities in the midgut of infected bees which leads to honey bee mortality (Naree et al., 2021c; Ponkit et al., 2021). My results showed that *N. ceranae* infection cause higher mortality in both *A. dorsata* and *A. mellifera* compared to healthy bees. Propolis and COS affected on the infectivity of *N. ceranae* in both honey bee species. The number of spores per bee (infectivity) of infected bees treated with 50% propolis extract or 0.5 ppm COS were lower than infected bees but not treated. Moreover, the infection rate and infection ratio of infected bees treated with propolis or COS were lower than infected not treated bees. Suggesting that propolis extract of stingless bee and COS might have toxic effects on *N. ceranae* spores. Previous research suggested that ethanolic propolis extract from stingless bee cause abnormal structure of *N. ceranae* spores resulting in interfering or inhibiting spore growth and development (Suwannapong et al., 2018; Yemor et al., 2016). Another research reported that chitosan (200 kDa, 75% deacetylated) is shown to have a preadaptive effect and increase the lifespan of honey bees due to the induction of protective antioxidant and immune mechanisms (Saltykova et al., 2016), suggesting that COS might protect the ventricular epithelial cells of honey bee midgut from *Nosema* invasion, and might has the efficacy to inhibit *Nosema* spore germination. Ke et al. (2022) reported that COS act on the cell wall and cell membrane of fungi by increased cellular permeability leading to leakage of cellular components. COS might act on N. ceranae like this.

In this study, infection by *N. ceranae* isolated from *A. mellifera* cause lower levels of trehalose, hypopharyngeal gland protein contents, and midgut proteolytic enzyme activities of infected bees, which corresponded with some studies (Naree et al., 2021c; Ponkit et al., 2021).

The trehalose levels, protein contents in hypopharyngeal glands, and midgut proteolytic enzyme activities in healthy bees were not affected by propolis extract and

COS, suggesting that treating with 50% propolis or 0.5 ppm COS in pollen is not having any effect for the honey bee health. In addition, propolis extract can increased the trehalose levels, protein content in hypopharyngeal gland, and activity of midgut proteolytic enzyme in infected bees, both *A. dorsata* and *A. mellifera*. The higher of trehalose levels and protein contents might be due to the lowering of the number of spores in infected bees treated with propolis extract or COS. Moreover, infected bees treated with propolis extract or COS also had lower infection ratio or lower infected-ventricular cells, resulting to have higher activity of midgut proteolytic enzyme. This will make infected bees treated with propolis or COS can digest and absorb the nutrients bester than not treated bees. Our results correspond to a previous study that showed 50% stingless bee propolis extract can improved health of both *N. ceranae*-infected *A. florea* and *A. dorsata* by increasing hemolymph trehalose levels and hypopharyngeal gland protein contents (Naree et al., 2021c; Suwannapong et al., 2018). Previous result also showed the efficiency of propolis and COS can increase the acini diameters of the hypopharyngeal glands of infected *A. dorsata*, that means their glands can synthesize more proteinaceous substance (Naree et al., 2021c).

N. ceranae infection suppressed the expression of antimicrobial peptide-encoding genes, hymenoptaecin in *A. dorsata* when measure on day 6 p.i. while up-regulation was found in *A. mellifera* on day 14 p.i. The expression of apidaecin and defensin-1 did not significantly change in both infected *A. dorsata* and *A. mellifera*. This is contrast to previous study that showed the expression of apidaecin, defensin-1, and hymenoptaecin were down-regulated in *A. mellifera* inoculated with *N. ceranae* (Chaimanee, Chantawannakul, Chen, Evans, & Pettis, 2012). Another study showed that the expression of defensin and hymenoptaecin were up regulated in *A. mellifera* after inoculated by *N. ceranae* for 4 and 7 days (Sinpoo et al., 2018). The differences of the results might be because of the different of the methodologies and type of tissues using for analysis.

Propolis treatment could elevate the expression of apidaecin and hymenoptaecin in *A. dorsata* after inoculated by *N. ceranae* for 6 days, and in *A. mellifera* after inoculated by *N. ceranae* for 3 days. The results also showed that COS treatment can elevated the expression of hymenoptaecin in *A. mellifera* infected by *N.*

ceranae at day 3 p.i. The expression of defensin-1 did not change in both infected bee species when treated with propolis or COS.

In conclusion, this study demonstrated that propolis and COS can improve health of *A. dorsata* and *A. mellifera* infection by *N. ceranae*, and can activate the infected bees to synthesize some antimicrobial peptides which might possibly use by honey bees to defense the *Nosema* infection.

Furthermore, it could advise beekeepers to provide propolis and COS as supplemental food for honey bees by mixing with sugar syrup or pollen to help improve their immune systems and reduce the impact of *Nosema* infections, perhaps leading to healthier and more productive hives. Moreover, supplying propolis and COS to managed honey bees can also reduce the opportunity to disseminate *Nosema* spores to wild bees which have overlapped in their foraging geographical area.

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APPENDIX

1. Reagents and solution for tissue staining using Periodic acid Schiff's reagent (PAS), counterstained with light green

1.1 Bouin's fluid fixative

Picric acid (saturated aqueous solution)	75	ml
Formaldehyde, 37-40%	25	ml
Glacial accetic acid	5	ml

1.2 Schiff's leuco-fuchsin solution

Basic fuchsin	1	g
Sodium bisulfate anhydrous	1	g
N-hydrochloric acid	10	ml
Distilled water	200	ml

1. One gram of basic fuchsin in 200 ml of hot distilled water. Lets it to boiling point and then cool to 50 °C.

2. The solution was then filtered using filter paper Whatmann no. 1 before adding 10 ml of N-hydrochloric acid.

3. one gram of sodium bisulfate anhydrous was added and homogenized. The solution was kept in dark at refrigerator for 48 h. until solution become straw colored.

1.3 0.5% Periodic acid solution

Periodic acid crystals	0.5	g
Distilled water	100	ml

1.4 N-hydrochloric acid solution

Hydrochloric acid (specific gravity: 1.19)	83.5	ml
Distilled water	916.5	ml

1.5 0.2% Light green counterstain

Fast green	0.2	g
Distilled water	100	ml
Glacial acetic acid	0.2	ml

1. 0.4 g of fast green was dissolved in hot distilled water, homogenized, and cooled down.

2. The solution was filtered using Whatmann no. 1 before adding 0.2 ml of glacial acetic acid.

3. The solution was then kept in a refrigerator.

2. Standard curve of trehalose measurement

The standard curve for trehalose measurement was generated using trehalose at concentration of 0, 1, 2, 4,6, and 8 µg per µl.

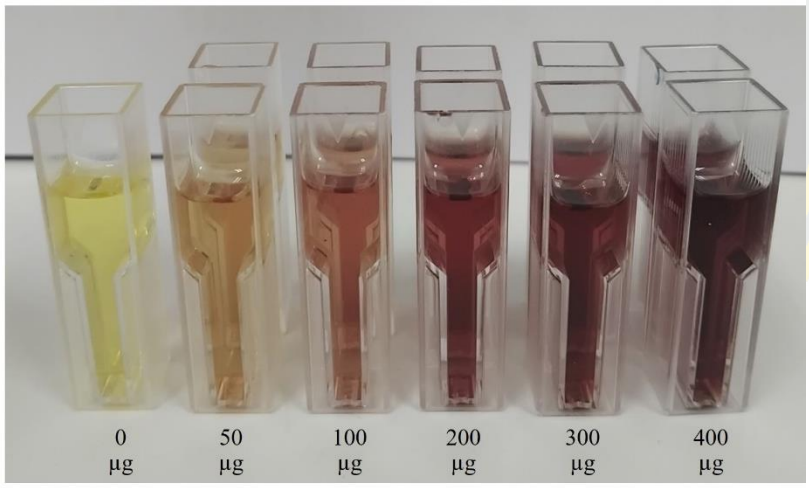

Figure A1 Shows the color range of the trehalose standard after boiling for 15 min and cooling for 20 min (0, 1, 2, 4, 6, and 8 µg per µl).

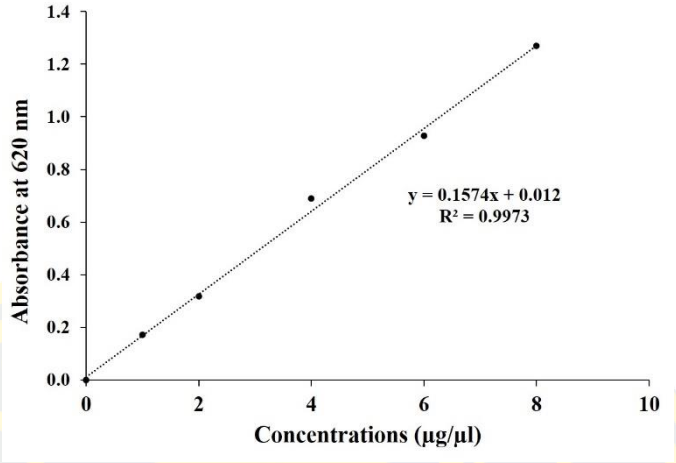

Figure A2 Shows the standard curve of trehalose at various concentrations (0, 1, 2, 4, 6, and 8 µg per µl).

3. Standard curve of protein measurement

The standard curve for protein measurement was generated using various concentrations (0, 250, 500, 750, 1,000, and 1,250 µg per ml) of bovine serum albumin (BSA).

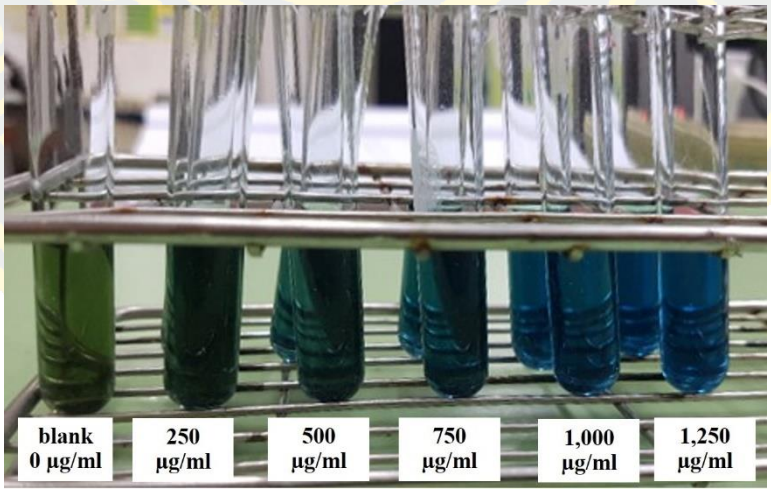

Figure A3 Shows the color range of standard protein (BSA) at different concentrations (0, 250, 500, 750, 1,000, and 1,250 µg/ml) after adding Coomassie brilliant blue G250 dye for 6 min.

.

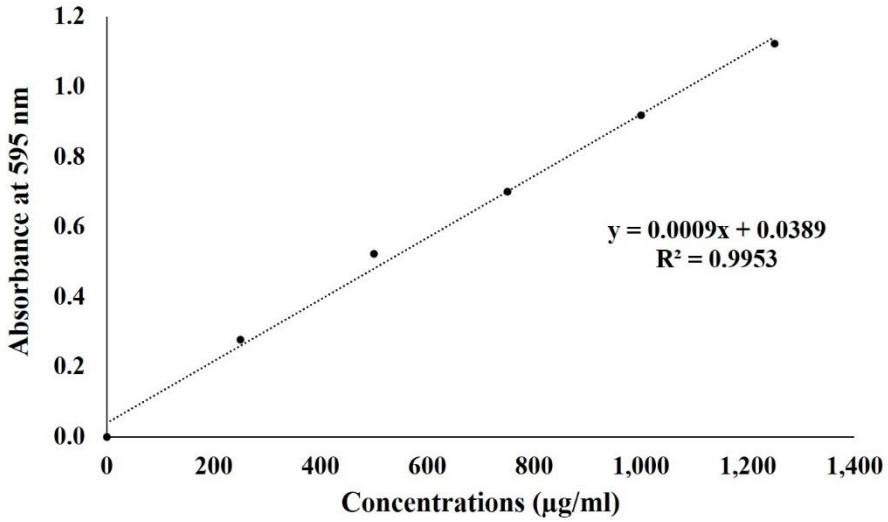

Figure A4 Shows a standard curve of protein (BSA) at various concentrations (0, 250, 500, 750, 1,000, and 1,250 µg/ml).

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